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METHODS AND COMPOSITIONS FOR INHIBITION OF HEPATIC
CLEARANCE OF TISSUE-TYPE PLASMINOGEN ACTIVATOR

Abstract:

Abstract of WO9414471

Methods and compositions for inhibiting the hepatic clearance of tissue-type plasminogen activator (t-PA) in vivo by administering a t-PA-hepatic clearance-inhibiting amount of 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment thereof, or genetically or chemically modified forms of the 39kDa protein or fragments thereof are described. Compositions for treatment of thrombolytic diseases comprised of t-PA and a t-PA-hepatic clearance-inhibiting effective amount of 39kDa protein, a t-PA-hepatic clearance inhibiting fragment thereof, and genetically or chemically modified forms of the 39kDa protein or its fragments are described.

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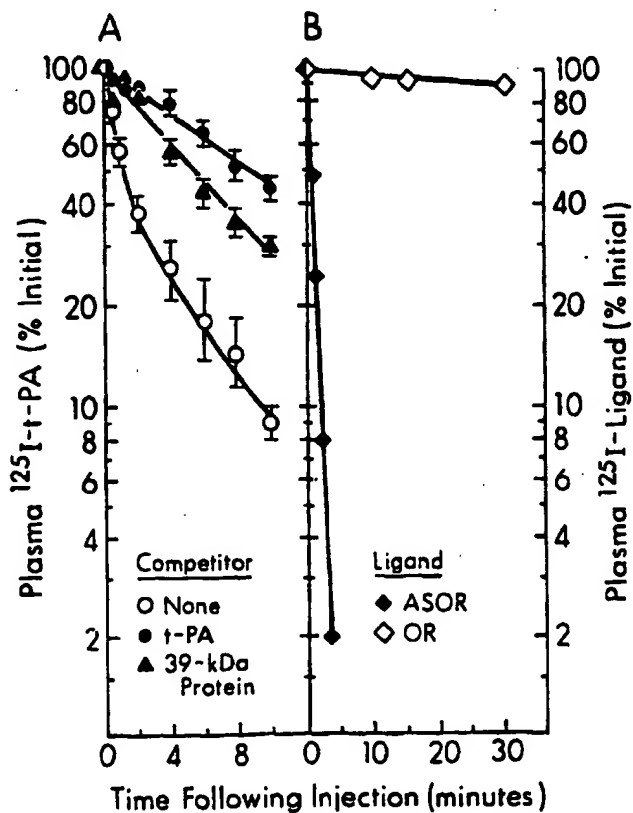
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(54) Title: METHODS AND COMPOSITIONS FOR INHIBITION OF HEPATIC CLEARANCE OF TISSUE-TYPE PLASMINOGEN ACTIVATOR

(57) Abstract

Methods and compositions for inhibiting the hepatic clearance of tissue-type plasminogen activator (t-PA) *in vivo* by administering a t-PA-hepatic clearance-inhibiting amount of 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment thereof, or genetically or chemically modified forms of the 39kDa protein or fragments thereof are described. Compositions for treatment of thrombolytic diseases comprised of t-PA and a t-PA-hepatic clearance-inhibiting effective amount of 39kDa protein, a t-PA-hepatic clearance inhibiting fragment thereof, and genetically or chemically modified forms of the 39kDa protein or its fragments are described.



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TITLE**METHODS AND COMPOSITIONS FOR INHIBITION OF
HEPATIC CLEARANCE OF TISSUE-TYPE PLASMINOGEN ACTIVATOR**TECHNICAL BACKGROUND

This invention was made with U.S. Government support under HL 17646 awarded by the National Institutes of Health. The U.S. Government has certain rights in the invention.

FIELD OF THE INVENTION

5 This invention relates to methods and compositions for inhibiting the hepatic clearance of tissue-type plasminogen activator (t-PA) *in vivo* comprising administering a t-PA-hepatic clearance-inhibiting amount of 39kDa protein or a t-PA-hepatic
10 clearance-inhibiting fragment thereof, or genetically or chemically modified forms of the 39kDa protein or fragments thereof; and to methods and compositions for treatment of thrombolytic diseases comprising administering t-PA and a t-PA-hepatic clearance-inhibiting effective amount of 39kDa protein,
15 a t-PA-hepatic clearance inhibiting fragment thereof, and genetically or chemically modified forms of the 39kDa protein or its fragments.

BACKGROUND OF THE INVENTION

20 Tissue plasminogen activator is an enzyme widely used as a thrombolytic agent in the treatment of acute myocardial infarction. t-PA is secreted from endothelial cells as a single polypeptide chain that is subsequently cleaved (between Arg₂₇₅ and Ile₂₇₆) into two chains held together by a single disulfide bond (Rijken, D.C. et al., (1981) J. Biol. Chem. 256, 7035-7041.
25 Both the single- and two-chain forms of the enzyme can bind to fibrin (Rijken, D.C. et al., (1982) J. Biol. Chem. 257, 2920-

2925; Higgins, D.L. et al., (1987) Biochem. 26, 7786-7791), although the two-chain form is catalytically more active (Wallen, P. et al., (1982) Biochim. Biophys. Acta 719, 318-328; Ranby, M., (1982) Biochim Biophys. Acta 704, 461-469; Tate, K.M. et al., (1987) Biochem. 26, 338-343; and Petersen, L.C. et al., (1988) Biochim. Biophys. Acta 952, 245-254). In addition, both forms of the enzyme (Jorgensen, M. et al., (1987) Thromb. Haemostasis 58, 872-878) are inactivated by a fast-acting plasminogen activator inhibitor (type 1) (PAI-1), a member of the serpin family that is secreted from endothelial cells and forms a covalent bond with Ser₄₇₈ of t-PA (Levin, E.G., (1983) Proc. Natl. Acad. Sci. USA 80, 6804-6808; and Sprengers, E.D. et al., (1987) Blood 69, 381-387).

Exogenously administered t-PA is capable of eliciting prompt thrombolysis in therapeutic doses that do not produce marked fibrinolysis in experimental animals with induced coronary artery thrombosis (Bergmann, S.R. et al., (1983) Science, 220, 1181-1183) and in patients with evolving myocardial infarction (Van de Werf, F. et al., (1984) New Eng. J. Med. 310, 609-613; Collen, D. et al., (1984) Circ. 70, 1012-1017; Van de Werf, F. et al., (1984) Circ. 69, 605-610; Thrombolysis in Myocardial Infarction Study Group, (1985) New Eng. J. Med. 312, 932-936; and European Cooperative Study Group, (1985) Lancet 1, 842-847). However, because the clearance of t-PA from the circulation is so rapid, continuous infusions have been required.

In vivo studies of t-PA clearance have been performed in a variety of species including mice (Fuchs, H.E. et al.,

(1985) Blood 65, 539-544); rats (Emeis, J.J. et al., (1985) Thromb. Haemost. 54, 661-664; Rijken, D.C. et al., (1986) Biochem. J. 238, 643-646; Kuiper, J. et al., (1988) J. Biol. Chem. 263, 18220-18224; Bakhit, C. et al., (1988) Fibrin. 2, 31-36; Krause, J. et al., (1990) Biochem. J. 267, 647-652); rabbits (Korninger, C. et al., (1981) Thromb. Haemostasis 46, 658-661; Bounameaux, H. et al., (1986) Blood 67, 1493-1497); dogs (Devries, S.R. et al., (1987) Fibrin. 1, 17-21; Yasuda, T. et al., (1988) J. Clin. Invest. 81, 1284-1291); and monkeys (Flameng, W. et al., (1985) J. Clin. Invest. 75, 84-90). These studies together with experiments in man (Garabedian, H.D. et al., (1986) Am. J. Cardiol. 58, 673-679; Verstraete, M. et al., (1986) Thromb. Haemostas. 56, 1-15) demonstrate the rapid removal of t-PA from the circulation, which varies from about $t_{1/2}$ = 1 min in rats to $t_{1/2}$ = 5 min in man.

The liver appears to be the major site of removal and catabolism of t-PA (Nilsson, T. et al., (1984) Scand. J. Haematol. 33, 49-53; Devries, S.R. et al., (1987) Fibrin. 1, 17-21; Korninger, C. et al., (1981) Thromb. Haemostasis 46, 658-661; Bounameaux, H. et al., (1986) Blood 67, 1493-1497; Beebe, D.P. et al., (1986) Thromb. Res. 43, 663-674; Nilsson, S. et al., (1985) Thromb. Res. 39, 511-521; Emeis, J.J. et al., (1985) Thromb. Haemost. 54, 661-664; Rijken, D.C. et al., (1986) Biochem. J. 238, 643-646; Fuchs, H.E. et al., (1985) Blood 65, 539-544; and Kuiper, J. et al., (1988) J. Biol. Chem. 263, 18220-18224). About 80% of exogenous t-PA delivered intravascularly rapidly accumulates in the liver and is subsequently degraded, with subsequent appearance of degradation

products in plasma. These studies support a general clearance mechanism for t-PA in which uptake and degradation within the liver is followed by the release of the degradation products initially into the blood and subsequently into the urine.

5 Further, the half-life of circulating t-PA is markedly prolonged in animals subjected to hepatectomy (Bounameaux, H. et al., (1986) Blood 67, 1493-1497; and Nilsson, T. et al., (1984) Scand. J. Haematol. 33, 49-53). Neither the protease active site nor a specific glycosylation pattern appears to be a major
10 determinant of hepatic recognition and degradation of t-PA in vivo (Fuchs, H.E. et al., (1985) Blood 65, 539-544), in perfused liver systems (Emeis, J.J. et al., (1985) Thromb. Haemost. 54, 661-664, or in isolated hepatocytes (Bakhit, C. et al., (1987) J. Biol. Chem. 262, 8716-8720. The clearance and catabolism of
15 t-PA has been reviewed in detail (Krause, J. (1988) Fibrin. 2, 133-142). However, information is limited regarding the particular cell type responsible for clearance of t-PA.

 After administration of fluorescent or radiolabelled t-PA to rats and subfractionation of the livers into
20 parenchymal, endothelial, and Kupffer cells, it was found that parenchymal and endothelial cells constitute the major sites for hepatic uptake (Fuchs, H.E. et al., (1985) Blood 65, 539-544; Sprengers, E.D. et al., (1987) Blood 69, 381-387; Kuiper, J. et al., Fibrin, 2:28 (1988) and Bugelski, P.J. et al., (1989)
25 Thromb. Res. 53, 287-303).

 The uptake of t-PA into all liver cell types is inhibited by in vivo competition with unlabelled t-PA whereas glycoproteins such as mannan and ovalbumin inhibit the specific

uptake of labelled t-PA in isolated liver endothelial cells. The endocytosis of t-PA is mediated, at least in part, by mannose receptors on endothelial cells (Einarsson, M. et al., (1985) *Thromb. Haemost.* 54, 270; and Kuiper, J. et al., (1988) *Fibrin.* 2, 28). Monensin, NH_4Cl , and cytochalasin B block the uptake and degradation of t-PA, indicating that the uptake is endocytotic and that the degradation is lysosomal. In hepatoma cell lines, representing parenchymal cells, t-PA clearance involves ligand binding, uptake, and degradation mediated by a high capacity, high-affinity specific receptor system (Owensby, D.A. et al., (1988) *J. Biol. Chem.* 263, 10587-10594). Subfractionation of rat liver parenchymal, endothelial, and Kupffer cells 5 minutes after ^{125}I -t-PA injection revealed that liver parenchymal cells are responsible for about 55% of the cleared ^{125}I -t-PA, endothelial cells for about 40%, and Kupffer cells for about 5% (Kuiper, J. et al., (1988) *J. Biol. Chem.* 263, 18220-18224; Rijken, D.C. et al., (1990) *Thromb. Res. Suppl.* X, 63-71).

Two distinct mechanisms for t-PA catabolism by hepatoma cells have been shown. t-PA complexed to PAI-1 is recognized by a PAI-1 dependent receptor on the cell surface of human hepatoma HepG2 cells (Schwartz, A.L. et al., (1981) *J. Biol. Chem.* 256, 8878-8881; Owensby, D.A. et al., (1988) *J. Biol. Chem.* 263, 10587-10594; Morton, P.A. et al., (1989) *J. Biol. Chem.* 264, 7228-7235; Bu, G. et al., (1992) *J. Biol. Chem.* 267, 15595-15602). t-PA in the absence of bioactive PAI-1 has been found to bind to PAI-1 independent receptors which mediate binding and endocytosis of t-PA on rat hepatoma MH_1C_1 cells (Bu,

G. et al., (1992) J. Biol. Chem. 267, 15595-15602) and on rat Novikoff hepatoma cells (Nguyen, G. et al., (1992) J. Biol. Chem. 267, 6249-6256). Although this PAI-1 independent t-PA clearance system has not been reported on human hepatocytes, the rapid clearance of intravenously injected t-PA, normally at a level far exceeding the available PAI-1, suggests the existence of a PAI-1 independent t-PA clearance system.

At present, t-PA is administered clinically in the form of an initial bolus that is followed by sustained infusion. The total amount of enzyme administered during a standard 3 hour treatment is 50-100 mg. Such large amounts are required for two reasons: first, to counterbalance the effects of the rapid clearance of t-PA from the circulation, and second, to overcome the effects of high concentrations of fast-acting inhibitors of the enzyme that are present in plasma and platelets. When high doses are used in an effort to increase the rate of clot lysis or to lyse refractory clots, there is a risk of systemic fibrinolysis which affects the body's capacity to stop bleeding and hemorrhage.

Reducing the rate of t-PA clearance from the circulation following administration would provide a significant advantage in clinical use. This would allow t-PA to be administered in much smaller doses than are currently required, thereby reducing, e.g., the risk of systemic fibrinolysis and hemorrhage. Accordingly, an objective of the invention is to provide methods and compositions for the inhibition of hepatic clearance of t-PA. Another objective of the invention is to provide a method and composition for the treatment of

thrombolytic diseases which allows t-PA to be administered in much smaller doses than currently required. These and other objectives and features of the invention will be apparent from the following description.

5

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a graph of the saturation binding of ^{125}I -39kDa protein to MH_2C_1 cells in the presence or absence of $0.5\mu\text{M}$ unlabelled 39kDa protein. (circles) = total ligand binding; (triangles) = non-specific ligand binding. Specific binding (squares) was calculated as the difference between total and non-specific binding. Each symbol represents the mean of triplicate determinations. The inset is a Scatchard plot of specific binding where B = bound ^{125}I -39kDa protein and B/F = bound/free ^{125}I -39kDa protein. FIG. 1B is a SDS-PAGE gel (10% acrylamide, non-reduced) of equivalent volumes of post-binding cell lysates, each from about 60,000 cells. The position of ^{125}I -39kDa protein is indicated by a closed arrow.

FIGS. 2A-B are graphs of the saturation binding of ^{125}I -t-PA and ^{125}I -39kDa protein to HepG2 cells. FIG. 2A is a graph of the saturation binding of ^{125}I -t-PA in the absence or presence of $1\mu\text{M}$ unlabelled t-PA. (squares) = total ligand binding; (triangles) = non-specific ligand binding; (circles) = specific binding calculated as the difference between total and non-specific binding. Symbols represent the means of triplicate determinations. The inset is a Scatchard plot of specific binding, where B = bound ^{125}I -t-PA, B/F = bound/free ^{125}I -t-PA. FIG. 2B is a graph of the saturation binding of the ^{125}I -39kDa

protein. The same symbols are used as in FIG. 2A. The inset, like FIG. 2A, is a Scatchard plot of specific binding.

FIGS. 3A-D are SDS-PAGE gels (8.5% acrylamide) of specific cross-linking of ^{125}I -39kDa protein to low density lipoprotein receptor-related protein (LRP) in the presence and absence of $0.5\mu\text{M}$ unlabelled 39kDa protein. In FIGS. 3A and 3B, MH_1C_1 cells were incubated in the presence of 0.5mM DTSSP for 1 hour. In FIGS. 3C and 3D, MH_1C_1 cells were incubated in the absence of 0.5mM DTSSP for 1 hour. Cells were then lysed and immunoprecipitated with normal rabbit serum (lanes 1 and 4), anti-LRP serum (lanes 2 and 5), or anti-39kDa protein serum (lanes 3 and 6). In FIGS. 3A and 3C, the SDS-PAGE gels were analyzed under nonreducing conditions. In FIGS. 3B and 3D, the SDS-PAGE gels were analyzed under reducing conditions. Gels from samples incubated in the presence of cross-linker were autoradiographed for 3 days at -70°C ; gels incubated in the absence of cross-linker were autoradiographed for only 2 days. The position of ^{125}I -39kDa protein (closed arrowhead) is indicated.

FIGS. 4A-B are SDS-PAGE gels of chemical cross-linking of ^{125}I -t-PA:PAI-1 to LRP on HepG2 cells. (N.R.) = normal rabbit serum; (α -t-PA) = anti-t-PA antibody; (α -PAI-1) = anti-PAI-1 antibody; (α -LRP) = anti-LRP antibody. FIG. 4A is a SDS-PAGE gel (7.5% acrylamide) analyzed under nonreducing conditions. FIG. 4B is a SDS-PAGE gel (7.5% acrylamide) analyzed under reducing conditions. The region of cross-linked material is marked with a bracket. The positions of ^{125}I -t-PA (closed arrow) and ^{125}I -t-PA:PAI-1 (open arrow) are indicated.

FIG. 5A is a graph of the inhibition of 3 nM ^{125}I -t-PA binding to HepG2 cells by the 39kDa protein in the absence or presence of increasing concentrations of competitor proteins. Each symbol represents the average of duplicate determinations and the standard deviations are less than 5%. FIG. 5B is a SDS polyacrylamide gel (7.5%) of post-binding buffers overlying cell monolayers and corresponding cell lysates where the gel was analyzed under nonreducing conditions using a sample buffer containing low concentration of SDS (0.2%). Samples were selected from either no competitor protein (lanes 1 and 5), or with 500 nM t-PA (lanes 2 and 6), 500 nM 39kDa protein (lanes 3 and 7), or 500 nM BSA (lanes 4 and 8).

FIG. 6 are SDS-PAGE gels of chemical cross-linking of t-PA:PAI-1 to LRP on ^{35}S -methionine-labelled HepG2 cells which were analyzed under nonreducing or reducing conditions. (N.R.) = normal rabbit serum (lanes 1, 6, 11); (α -t-PA) = anti-t-PA antibody (lanes 2, 7, 12); (α -PAI-1) = anti-PAI-1 antibody (lanes 3, 8, 13); (α -39kDa) = anti-39kDa protein antibody (lanes 4, 9, 14); (α -LRP) = anti-LRP antibody (lanes 5, 10, 15). The regions of cross-linked material in the non-reducing gel are marked with brackets. The following positions are indicated: LRP large subunit (515 kDa) (2 closed arrowheads); LRP small subunit (85 kDa) (1 closed arrowhead); PAI-1 (1 closed arrow); and t-PA:PAI-1 complex (1 open arrow).

FIGS. 7A-B are SDS-PAGE gels (8.5% acrylamide) of cross-linking of unlabelled 39kDa protein to the cell surface of metabolically labelled rat MH_2C_1 cells. Cell monolayers in lanes 4-6 were incubated for 1 hour with 0.5 mM DTSSP, whereas cell

monolayers in lanes 1-3 were incubated for 1 hour in PBSc alone. Cells were then lysed and immunoprecipitated with either normal rabbit serum (lanes 1 and 4), affinity purified α -rat 39kDa protein (lanes 2 and 5), or affinity purified α -human LRP (lanes 3 and 6). In FIG. 7A, the SDS-PAGE gel was analyzed under nonreducing conditions. In FIG. 7B, the SDS-PAGE gel was analyzed under reducing conditions. The *closed arrowhead* indicates the position of the 39kDa protein. The *open* and *closed arrows* indicate, respectively, the positions of the 85kDa and 520kDa subunits of LRP.

FIGS. 8A-F are SDS-PAGE gels (8.5% acrylamide) of co-binding and cross-linking of ^{125}I -39kDa protein and unlabelled t-PA to rat MH_1C_1 cells in the presence or absence of 0.5 μM unlabelled 39kDa protein. In FIGS. 8A, 8C, and 8E, the SDS-PAGE gels were analyzed under nonreducing conditions. In FIGS. 8B, 8D, and 8F, the SDS-PAGE gels were analyzed under reducing conditions.

FIG. 9 is a graph of rapid endocytosis and degradation of surface bound ^{125}I -39kDa protein by MH_1C_1 cells in the presence and absence of 0.5 μM unlabelled 39kDa protein. (*circles*, degraded ligand) = TCA soluble radioactivity; (*diamonds*, both dissociated and degraded ligand) = total radioactivity; (*squares*, plasma membrane associated) = Pronase sensitive ligand; (*triangles*, cell associated) = Pronase resistant ligand. Each data point represents specific radioactivity (i.e., the difference of total and non-specific radioactivity) and is the mean of triplicate determinations.

FIGS. 10A-B are graphs of the inhibition of ^{125}I -t-PA uptake and degradation by the 39kDa protein on HepG2 cells. (open squares) = prewarmed (37°C) binding buffer containing ^{125}I -t-PA (3 nM); (open circles) = prewarmed (37°C) binding buffer containing ^{125}I -t-PA (3 nM) in the presence of 1 μM concentration of t-PA; (open triangles) = prewarmed (37°C) binding buffer containing ^{125}I -t-PA (3 nM) in the presence of 1 μM concentration of the 39-kDa protein; (closed circles) = prewarmed (37°C) binding buffer containing ^{125}I -t-PA (3 nM) in the presence of 1 μM concentration of BSA. FIG. 10A shows TCA soluble radioactivity representing the degraded ligands in fmoles equivalents of t-PA. FIG. 10B shows the radioactivity associated with each cell lysate in fmoles equivalents of t-PA. In each graph, each symbol represents the average of triplicate determinations and the standard deviations were less than 5%.

FIGS. 11A-B are graphs of the inhibition of ^{125}I -t-PA binding by rat 39kDa protein on MH_1C_1 cells in the presence of increasing concentrations of either unlabelled t-PA or 39kDa protein. Each point represents the mean of triplicate determinations. FIG. 13A shows the inhibition of ^{125}I -t-PA binding by the 39kDa protein. FIG. 13B shows the inhibition of ^{125}I -t-PA binding by unlabelled t-PA.

FIG. 12 is a graph of the rapid uptake and degradation of ^{125}I -39kDa protein by rat MH_1C_1 cells at 37°C. Closed circles represent TCA soluble counts (internalized and degraded ligand) in the extracellular media. Triangles indicate the amount of cell associated radioactivity as determined following lysis of the cell monolayers. Open circles represent the sum of both

cell associated and extracellular degraded ligand. Each point is the mean of triplicate determinations.

FIG. 13 is a graph of the inhibition of the ^{125}I -39kDa protein by t-PA. Each symbol represents the average of
5 duplicate determinations and the standard deviations are less than 5%.

FIG. 14 is a graph of the inhibition of 3 nM ^{125}I -t-PA binding to HepG2 cells by anti-LRP antibody. (*open circles*) = rabbit non-immune IgG; (*closed circles*) = rabbit polyclonal
10 anti-LRP IgG. Each symbol represents the average of duplicate determinations and the standard deviations are less than 5%.

FIG. 15A is a Coomassie-stained SDS-PAGE gel of purified 39kDa protein (10 μg). FIG. 15B is a graph of the inhibition of ^{125}I -t-PA binding to rat liver MH_1C_1 cells by 39kDa
15 protein.

FIGS. 16A-B are graphs of the plasma clearance of ^{125}I -t-PA in rat *in vivo* in the absence or presence of unlabelled t-PA or 39kDa protein. Bars = S.E.M. FIG. 16A shows the plasma clearance of ^{125}I -t-PA alone (n=8) (*open circles*); the plasma
20 clearance of ^{125}I -t-PA when 12 nmol unlabelled t-PA is administered prior to ^{125}I -t-PA (n=3) (*closed circles*); and the plasma clearance of ^{125}I -t-PA when 250 nmol unlabelled 39kDa protein is administered prior to ^{125}I -t-PA (n=4) (*closed triangles*). FIG. 16B shows the plasma clearance of control
25 proteins ^{125}I -asialorosomucoid (*closed diamonds*) and ^{125}I -orosomucoid (*open diamonds*).

FIG. 17 is a graph of the plasma clearance of ^{125}I -39kDa protein in rat *in vivo* in the presence of unlabelled 39kDa

protein. (O) = administration of ^{125}I -39kDa protein alone (n=6);
(●) = administration of 12.5 nmol unlabelled 39kDa protein prior
to ^{125}I -39kDa protein (n=4); (■) = administration of 50 nmol
unlabelled 39kDa protein prior to ^{125}I -39kDa protein (n=4); (▲) =
5 administration of 125 nmol unlabelled 39kDa protein prior to
 ^{125}I -39kDa protein (n=2).

FIG. 18 is a graph of the inhibition of ^{125}I -t-PA
binding to rat liver MH_2C_1 cells by intact 39kDa protein, 20kDa
N-terminal fragment and 28kDa C-terminal fragment. (●) = intact
10 39kDa protein; (■) = the 20kDa N-terminal fragment; (▲) = the
28kDa C-terminal fragment; and (O) = PVDF-elution buffer alone.

SUMMARY OF THE INVENTION

The present invention provides a method of inhibiting
the hepatic clearance of t-PA *in vivo* in humans by the
15 administration of a t-PA-hepatic clearance-inhibiting amount of
a 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment
thereof. The 39kDa protein and fragments thereof may be
genetically or chemically modified.

An advantage of this method is that the plasma half-
20 life of t-PA is significantly increased.

The present invention also provides a 28kDa protein
and a chemically synthesized gene encoding this protein. It has
been found that this 28kDa protein inhibits hepatic clearance of
t-PA.

25 The present invention also provides a method of
thrombolysis in a mammal by the administration of a
thrombolytically effective amount of t-PA and a t-PA-hepatic

clearance-inhibiting effective amount of 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment thereof.

An advantage of this method is that the inhibition of hepatic clearance of t-PA enables smaller doses of t-PA to be
5 used to achieve the same level of thrombolysis.

The present invention further provides a pharmaceutical composition for a mammalian patient comprising t-PA and a t-PA- hepatic clearance-inhibiting amount of 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment thereof.

10 An advantage of this pharmaceutical composition is that the inclusion of a t-PA-hepatic clearance-inhibiting protein enables a smaller dosage amount of t-PA to be used with both physiological and cost benefits for the patient.

The present invention also provides a composition for
15 treating thrombolytic diseases in a mammal comprising t-PA and a t-PA-hepatic clearance-inhibiting amount of 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment thereof.

An advantage of this composition is that the amount of t-PA effective for treating thrombolytic diseases is markedly
20 reduced in comparison to current requirements.

Upon further study of the specification and appended claims, further objectives and advantages of this invention will become apparent to those skilled in the art.

DETAILED DESCRIPTION OF THE INVENTION

25 It is to be understood that while the present invention is primarily contemplated for humans, it is also contemplated for use in veterinary medicine.

In accordance with the invention, a 39kDa protein which competitively binds the hepatic receptor for t-PA has been found. By binding to the hepatic receptor for t-PA, the 39kDa protein prevents t-PA from being bound to the receptor and removed from the circulating plasma by endocytosis. This increases the plasma half-life of t-PA thereby prolonging t-PA's therapeutic effectiveness. An increase in the plasma half life of t-PA means that a smaller amount of t-PA may be used, which reduces the risk of systemic fibrinolysis and hemorrhage. As t-PA is very expensive, a significant cost savings can be achieved which, in turn, increases the availability of t-PA for clinical use.

The 39kDa protein is an active, effective, competitive binding agent for the hepatic receptor for t-PA. This t-PA-hepatic clearance-inhibiting protein is characterized by binding to LRP and inhibiting the binding of t-PA to LRP up to about 80% (K_i of about 0.5 nM) (see Examples 5-8 and 10, *infra*). Fragments of this 39kDa protein, particularly a 28kDa protein fragment, also competitively bind to the t-PA hepatic receptor. When the 39kDa protein or t-PA-hepatic clearance inhibiting fragment thereof is employed in the present invention, the standard dose of t-PA is reduced up to 90%.

Hepatic clearance of t-PA is inhibited *in vivo* in humans by administering a t-PA-hepatic clearance-inhibiting amount of the 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment of the 39kDa protein. The mode of administration is preferably intravenous. The preferred amount of 39kDa protein or fragment administered to the human to

inhibit hepatic clearance is in the range of about 60 to 6,000 mg/kg of body weight/dose. When the fragment of the 39kDa protein is the 28kDa protein, the preferred amount administered to the human to inhibit hepatic clearance is in the range of about 38 to 3,800 mg/kg of body weight/dose. The 39kDa protein or its fragments may be administered to the human concurrently with t-PA, but is preferably administered up to 20 minutes prior to the administration of t-PA. Administration of the 39kDa protein or its fragments reduces hepatic clearance of t-PA between 20 and 100%. The reduction in hepatic clearance is measured by the increase in plasma half-life of t-PA.

It is to be understood that modified forms of the 39kDa protein and fragments thereof which inhibit hepatic clearance of t-PA that are made by chemically or genetically modifying the amino acid sequence of the 39kDa protein or its fragments are encompassed within the scope of the present invention. Such modified forms are characterized by their ability to bind to LRP and to reduce hepatic clearance of t-PA between 20 and 100%.

The 39kDa protein of the present invention has the following amino acid sequence set forth in the Sequence Listing as SEQ ID NO: 1:

```

1           10           20           30
Y S R E K N Q P K P S P K R E S G E E F R M E K L N Q L W E K A Q
25           40           50           60
R L H L P P V R L A E L H A D L K I Q E R D E L A W K K L K L D G
           70           80           90
L D E D G E K E A R L I R N L N V I L A K Y G L D G K K D A R Q V
100          110          120          130
30 T S N S L S G T Q E D G L D D P R L E K L W H K A K T S G K F S G
```

140 150 160
E E L D K L W R E F L H H K E K V H E Y N V L L E T L S R T E E I
170 180 190
H E N V I S P S D L S D I K G S V L H S R H T E L K E K L R S I N
5 200 210 220 230
Q G L D R L R R V S H Q G Y S T E A E F E E P R V I D L W D L A Q
240 250 260
S A N L T D K E L E A F R E E L K H F E A K I E K H N H Y Q K Q L
10 270 280 290
E I A H E K L R H A E S V G D G E R V S R S R E K H A L L E G R T
300 310 320
K E L G Y T V K K H L Q D L S G R I S R A R H N E L

The 39kDa protein was prepared as shown in Example 1.

EXAMPLE 1

15 Purification of the 39kDa Protein

The procedure for purification of the 39kDa protein from strains of *E.coli* carrying the over-expression plasmid *pGEX-39kDa* has been described in Herz, J. et al., (1991) *J. Biol. Chem.* 266, 21232-21238. A modified version of that
20 procedure, described below, was employed.

Cultures of *E.coli* strain DH5 α carrying the over-expression plasmid *pGEX-39kDa* were grown to mid-log phase in LB medium with 100 μ g/ml ampicillin at 37°C. Cultures were cooled to 30°C and supplemented with 0.01%
25 isopropylthio- β -D-galactoside to induce expression of the glutathione-S-transferase-39kDa fusion protein. Following a 4-6 hour induction at 30°C, cultures were cooled on ice and collected by centrifugation.

All of the following steps were carried out at 4°C.
30 Cell pellets were lysed in PBSa with 1% Triton X-100, 1 μ M pepstatin, 2.5 μ g/ml leupeptin, 0.2 mM phenylmethylsulfonyl

fluoride (PMSF), and 1 μ M ethylenediaminetetraacetate (EDTA). Sonication of this lysate was performed using a Branson Model 450 Sonifier, with the resulting membranes and other cellular debris collected by centrifugation at 15,000 g for 15 minutes.

5 The supernatant from this step was incubated overnight with agarose immobilized glutathione beads (Sigma Chemical Co.) in PBSa and 0.1% sodium azide. The beads were then washed, and elution of the fusion protein was carried out by competition with 5 mM reduced glutathione (Sigma Chemical Co.). Following
10 dialysis, the fusion protein was cleaved by an overnight incubation with 100 ng of activated human thrombin per 50 μ g of fusion protein. The glutathione-S-transferase epitope was subsequently removed by further incubation with agarose immobilized glutathione beads.

15

* * * * *

The 28kDa protein fragment of the 39kDa protein ("28kDa protein") of the present invention has the following amino acid sequence set forth in the Sequence Listing as SEQ ID NO: 2:

20 1 10 20 30
P R L E K L W H K A K T S G K F S G E E L D K L W R E F L H H K
40 50 60
E K V H E Y N V L L E T L S R T E E I H E N V I S P S D L S D I K
25 70 80 90
G S V L H S R H T E L K E K L R S I N Q G L D R L R R V S H Q G Y
100 110 120 130
S T E A E F E E P R V I D L W D L A Q S A N L T D K E L E A F R
140 150 160
E E L K H F E A K I E K H N H Y Q K Q L E I A H E K L R H A E S V
30 170 180 190
G D G E R V S R S R E K H A L L E G R T K E L G Y T V K K H L Q D

200 209
L S G R I S R A R H N E L

The 28kDa protein is characterized by a molecular weight of 28,000 daltons on SDS-PAGE, stability to acid hydrolysis, solubility in 1% Triton X-100, and having approximately the same inhibitory activity (K_i) on t-PA binding to the hepatic receptor as the 39kDa protein. The 28kDa protein may be cloned and purified as shown in the following example.

EXAMPLE 2

10 Cloning of the 28kDa Protein

The 28kDa protein is produced with a bacterial expression system. The gene encoding this protein is synthesized using polymerase chain reaction (PCR) with the following primers set forth in the Sequence Listing as SEQ ID NO: 3 and SEQ ID NO: 4, respectively:

5'CCGCGTGGATCCCCCAGGCTGGAAAAGCTGTGG3',

5'TCAATGAATTCTCAGAGTCGCTCGCCGTCGCCCAC3'.

These PCR primers contain built-in restriction sites (BamH1 and EcoR1, respectively). The PCR product after restriction enzyme digestion is cloned directly to the pGEX-2T vector (Pharmacia). Other bacterial expression vectors may be used. The constructed plasmid is used to transform bacteria *E. coli* strain DH5 α F' and this bacterial transformant bearing the recombinant plasmid is used to produce the 28kDa protein using the procedure of Example 1.

* * * * *

Using standard recombinant techniques, a chemically synthesized gene encoding the 28kDa protein may be prepared. The chemically synthesized gene comprises a chemically

synthesized polynucleotide which codes on expression for the amino acid sequence of the 28kDa protein given above.

A rat protein has also been found which binds to LRP and inhibits binding of t-PA to the LRP hepatic receptor. This
5 rat protein has the following amino acid sequence set forth in the Sequence Listing as SEQ ID NO: 5:

```

1          10          20          30
P R L E K L W H K A K T S G S V R L T S C A R V L H K E K I H E Y

          40          50          60
10 N V L L D T L S R A E E G Y E N L L S P S D M T H I K S D T L A S

          70          80          90
K H S E L K D R L R S I N Q G L D R L R K V S H Q L R P A T E F E

          100          110          120          130
E P R V I D L W D L A Q S A N F T E K E L E S F R E E L K H F E A

          140          150          160
15 K I E K H N H Y Q K Q L E I S H Q K L K H V E S I G D P E H I S R

          170          180          190
N K E K Y V L L E E K T K E L G Y K V K K H L Q D L S S R V S R A

          200
20 R H N E L
```

Using standard recombinant techniques, a chemically synthesized gene encoding this rat protein may be prepared. The chemically synthesized gene comprises a chemically synthesized polynucleotide which codes on expression for the amino acid
25 sequence of the rat protein given above.

A method of thrombolysis in a mammal is also provided. In accordance with the invention, the method utilizes the t-PA hepatic clearance-inhibiting effect of the 39kDa protein and fragments thereof. The method comprises administering to the
30 mammal, preferably intravenously, a thrombolytically effective amount of t-PA and a t-PA-hepatic clearance-inhibiting effective amount of the 39kDa protein or a t-PA-hepatic clearance-

inhibiting fragment thereof. The preferred amount of t-PA is between 0.15 and 1.5 mg/kg of body weight/dose. The preferred amount of 39kDa protein or fragment administered to the mammal is between 60 to 6,000 mg/kg of body weight/dose. When the
5 fragment of the 39kDa protein is the 28kDa protein, the preferred amount administered to the mammal is between 38 to 3,800 mg/kg of body weight/dose. The 39kDa protein or fragment thereof may be administered to the mammal up to 20 minutes prior to administering the t-PA.

10 A composition for treating thrombolytic diseases which may be employed in the method of thrombolysis includes an effective amount of t-PA and an effective amount of a t-PA hepatic clearance-inhibiting 39kDa protein or a t-PA hepatic clearance-inhibiting fragment thereof. The t-PA is preferably
15 present in a dosage amount of from about 0.15 to 1.5 mg/kg of body weight/dose. The 39kDa protein or fragment thereof is present in a dosage amount of from about 60 to 6,000 mg/kg of body weight/dose. When the fragment of the 39kDa protein is the 28kDa protein, the dosage amount is from about 38 to 3,800 mg/kg
20 of body weight/dose.

t-PA is primarily used in the treatment of thrombolytic diseases, but t-PA has also been used for treating myocardial infarctions, tumors via fibrinolysis, and sickle cell anemia via fibrinolysis. The present invention accordingly
25 provides a pharmaceutical composition for a mammalian patient containing t-PA, the improvement comprising further including a t-PA hepatic clearance-inhibiting amount of 39kDa protein or a t-PA hepatic clearance-inhibiting fragment thereof. The t-PA is

preferably present in a dosage amount of from about 0.15 to 1.5 mg/kg of body weight/dose. The 39kDa protein or fragments thereof is present in a dosage amount of from about 60 to 6,000 mg/kg of body weight/dose. When the fragment of the 39kDa protein is the 28kDa protein, the dosage amount is from about 38 to 3,800 mg/kg of body weight/dose.

The ability of the 39kDa protein to bind to the t-PA hepatic receptor is demonstrated in the following examples.

EXAMPLE 3

10 Specific Binding of the 39kDa Protein to the MH₁C₁ Cell surface

Rat MH₁C₁ hepatoma cells are known to specifically bind ¹²⁵I-t-PA with high affinity to the cell surface ($K_d = 4.9 \pm 1.3$ nM (SD); $B_{max} = 78,000 \pm 35,000$ (SD)) (Bu, G. et al., J. Biol. Chem. 267, 15595-15602). A specific interaction between the 39kDa protein and rat MH₁C₁ hepatoma cells was demonstrated by the following saturation binding analyses.

Cell Culture and Media

Rat MH₁C₁ hepatoma cells were grown in Eagle's minimum essential media (MEM) with Earle's salts (Gibco BRL) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in humidified air containing 5% CO₂ as described in Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602. Cell monolayers were used at approximately 90% confluence, and the media was replaced the day prior to each experiment.

Radiolabelling of purified proteins

Recombinant 39kDa protein and t-PA were radiolabelled with carrier-free sodium ¹²⁵I-iodide (DuPont New England Nuclear

Products) using the Iodogen procedure described in Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602. Specific radioactivities for each radiolabelled protein were between 1 and 2×10^7 cpm/ μ g of protein as measured by γ scintillation spectrometry.

Saturation Binding Analysis

Cells were grown in 12 well dishes to approximately 10^6 cells per well. Monolayers were taken directly from 37°C and cooled on ice. The binding buffer used for the recombinant 39kDa protein was PBSc (phosphate-buffered saline supplemented with 1 mM CaCl_2 and 0.5 mM MgCl_2). Each cell monolayer was washed three times with PBSc prior to the addition of binding buffer containing various concentrations of radiolabelled ^{125}I -39kDa protein, either in the presence or absence of an excess of unlabelled protein. Cell monolayers were incubated at 4°C with between 1 and 24 nM recombinant ^{125}I -39kDa protein, either in the presence or absence of 0.5 μ M unlabelled protein.

Following a 90 minute incubation, cell monolayers were washed three additional times with PBSc to remove non-specifically associated ligand, and lysed in "low SDS lysis buffer" (0.0625 M Tris-HCl, pH 6.8, with 0.2% sodium dodecyl sulfate, 10% glycerol, and 0.01% bromophenol blue), as described in Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602. The amount of cell associated ligand was determined by γ scintillation spectrometry. In cases where t-PA and the 39kDa protein were co-bound, the t-PA binding buffer (PBSa containing 0.2 mM CaCl_2 and 10 mM ϵ -amino-n-caproic acid) was used as

described in Bu, G. et al., (1992) J. Biol. Chem. 267,
15595-15602.

As shown in FIG. 1A, non-specific binding of ^{125}I -39kDa
protein increased linearly over the range of concentrations
5 tested, while total binding increased in a curvilinear fashion,
approaching an asymptote above 12 nM. Scatchard analysis (23)
(inset to FIG. 1A) is consistent with a single homogeneous
population of binding sites and yields an equilibrium
dissociation constant (K_d) of 3.2 nM with 220,000 binding sites
10 per cell, for this particular experiment.

The results from ten independent binding experiments
indicate a mean K_d value of 3.3 ± 0.9 (SD) nM for ^{125}I -39kDa
protein binding to MH_1C_1 cells, with an average of $380,000 \pm$
190,000 (SD) binding sites per cell, as shown in the following
15 table, Table 1. A represents the mean values with standard
deviations for binding of both the 39kDa protein and t-PA to
 MH_1C_1 cells. The cumulative data from all independent binding
experiments was used. B represents the mean values with
standard deviation from only simultaneously performed binding
20 experiments. C represents data derived from simultaneous
binding experiments performed using either untreated MH_1C_1 cells,
or cells pre-incubated for 30 minutes with 0.1% saponin at 4°C.

Table 1

Specific Binding of ^{125}I -39kDa Protein and ^{125}I -t-PA to MH_1C_1 Cells

ligand	preincubation	$B_{\text{max}} \pm \text{S.D.}$ (10^3 sites/cell)	$K_d \pm \text{S.D.}$ (nM)	Experiment (n)
A. Summary				
^{125}I -39kDa	none	380 ± 190	3.3 ± 0.9	10
^{125}I -t-PA	none	78 ± 35	4.9 ± 1.3	7
B. Simultaneous Assays				
^{125}I -39kDa	none	390 ± 150	3.5 ± 0.9	4
^{125}I -t-PA	none	60 ± 35	5.6 ± 1.5	4
C. Simultaneous Assays Performed in the Presence or Absence of Saponin				
^{125}I -39kDa	none	250 ± 8	2.7 ± 1.3	3
^{125}I -t-PA	saponin	515 ± 24	9.1 ± 3.6	3

These data indicate both specific and saturable binding of the 39kDa protein to a single kinetic species of high affinity receptor on the MH_1C_1 cell surface.

The nature of the 39kDa protein binding species was shown by visualizing cell lysates from each binding experiment by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (FIG. 1B). Under the mild conditions used in these experiments (0.2% SDS), the 39kDa protein appeared to bind in isolation, without apparent association with any auxiliary binding proteins. These experiments demonstrate that there is a specific and saturable interaction between the 39kDa protein and an MH_1C_1 cell surface molecule(s).

EXAMPLE 4**Specific Binding of ^{125}I -t-PA and
 ^{125}I -39kDa Protein to HepG2 Cells**

The following saturation binding analyses

5 demonstrated that there is a specific interaction between the 39kDa protein and human hepatoma HepG2 cells. Additionally, the binding parameters to human hepatoma HepG2 cells of ^{125}I -t-PA and ^{125}I -39kDa protein were compared by parallel saturation binding experiments.

10 Binding studies were carried out at 4°C to avoid possible ligand uptake and degradation. Binding buffer for ^{125}I -t-PA included 10 mM EACA since this lysine analog reduced nonspecific ^{125}I -t-PA binding (presumably to lysine residues on the cell surface) without affecting specific ^{125}I -t-PA binding.

15 (See, Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602). Throughout this example, protein concentration was determined using a Bio-Rad protein assay with bovine serum albumin as a standard. Cell number was determined by counting cells with a hemocytometer.

20 Cell Culture

Human hepatoma HepG2 cells were maintained in culture as described in (Schwartz, A. L. et al., (1981) J. Biol. Chem. 256, 8878-8881). Cells were cultured in Earle's minimum essential medium (MEM) with glutamine (Gibco

25 Laboratories) supplemented with 10% (v/v) fetal calf serum (Gibco Laboratories), penicillin (100 units/ml), streptomycin (100 µg/ml), and were incubated at 37°C in humidified air containing 5% CO₂. Cultures were supplemented with fresh media

12 hours prior to use. Cell monolayers were generally cultured for two days before use at 80-90% confluence.

Production and Isolation of 39kDa Protein

The 39kDa protein was produced and isolated in accordance with the procedure of Example 1.

Protein Iodination

Iodination of t-PA and 39kDa protein were performed using IODOGEN (Pierce Chemical Co.) as described in Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602. The specific activity was generally 5-10 $\mu\text{Ci}/\mu\text{g}$ of protein as determined by γ scintillation spectrometry. The unincorporated ^{125}I after gel-filtration purification over a PD-10 column (Pharmacia) was less than 2% of the total radioactivity.

Saturation Binding Assays

Cells were seeded into multiwell (12 wells/plate) disposable plastic tissue culture plates 2 days prior to assay. Ligand binding buffer for t-PA was composed of phosphate-buffered saline (PBS) supplemented with 0.2 mM CaCl_2 and 10 mM EACA, whereas PBSc (PBS containing 1 mM CaCl_2 and 0.5 mM MgCl_2) was used for 39kDa protein binding. Binding experiments were performed at 4°C to prevent possible concomitant internalization during the binding interval. Cell monolayers were washed three times on ice with prechilled binding buffer. Binding was initiated by adding 0.5 ml binding buffer containing selected concentrations of ^{125}I -labelled ligand in the absence or presence of an excess unlabelled ligand (1 μM). After incubation at 4°C for 1.5 hours, buffer containing unbound ligand was removed. Cells

were then washed three times with binding buffer and lysed in 0.0625 M Tris-HCl, pH 6.8, containing 0.2% (w/v) SDS and 10% (v/v) glycerol ("low-SDS lysis buffer"). Radioactivity of cell lysates was quantified by γ scintillation spectrometry.

5 For some experiments, cell lysates were further analyzed by SDS-PAGE and autoradiography as described below. Total binding was determined in the presence of ^{125}I -labelled ligand alone. Nonspecific binding was determined in the presence of excess unlabelled ligand. Specific ligand binding was defined
10 as the difference between total and nonspecific binding.

Specific binding of ^{125}I -t-PA over the concentration range of 0.1-48 nM yielded a curvilinear plot. FIG. 2A shows a representative experiment from seven such experiments performed. Scatchard analysis of the binding data showed a
15 curvilinear plot concave upwards (inset, FIG. 2A). This result may be due to either the presence of multiple classes of binding sites with different but fixed affinities, or the existence of site-site interactions of the type defined as "negative cooperativity". (See Meyts, P. D. et al., (1976) J.
20 Biol. Chem. 251, 1877-1888). Due to the type of saturation binding experiments performed, these two possibilities could not be distinguished. When multiple classes of binding sites are assumed, an estimate of two binding sites best fit the data. From the Scatchard analysis shown in FIG. 2A, 40,300
25 sites are estimated for the high affinity binding ($K_d = 1.6$ nM) and 146,200 sites ($K_d = 32.5$ nM) for the low affinity binding. When cell lysates from selected ligand concentrations were analyzed via SDS-PAGE, specific binding occurred predominantly

in the form of the ^{125}I -t-PA:PAI-1 complex. This is consistent with PAI-1-dependent ^{125}I -t-PA binding on HepG2 cells. (See, Morton, P. A. et al., (1989) J. Biol. Chem. 264, 7228-7235; Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602).

5 Specific binding of ^{125}I -39kDa protein to HepG2 cells was performed over the concentration range of 1-24 nM. As shown in FIG. 2B, the 39kDa protein specifically bound to HepG2 cells with a low level of non-specific binding. Saturation of specific binding was observed at ^{125}I -39kDa
10 protein concentrations in excess of 12 nM. Scatchard analysis of the binding data from five such experiments yielded $197,000 \pm 23,000$ (S.D.) homogeneous high affinity surface binding sites per cell with an apparent $K_d = 5.1 \pm 0.8$ nM (S.D.) (166,000 binding sites per cell and $K_d = 4.9$ nM for the
15 experiment shown in FIG. 2B). When post-binding cell lysates were analyzed by SDS-PAGE, ^{125}I -39kDa protein was observed at its native molecular mass (i.e., 39kDa) indicating that the binding did not include an SDS-stable complex with other proteins.

20

* * * * *

The following example demonstrates that low density lipoprotein receptor related protein (LRP) is a receptor for the 39kDa protein.

EXAMPLE 5

25 Cross-linking of ^{125}I -39kDa Protein to the MH_2C_1 Cell Surface

The 39kDa protein was originally isolated via its ability to co-purify with a low density lipoprotein receptor related protein (LRP) by $\alpha 2$ -macroglobulin affinity

chromatography. See Ashcom, J.D. et al., (1990) J. Cell Biol. 110, 1041-1048; Jensen, P.H. et al., (1989) FEBS Lett. 255, 275-280. This low density lipoprotein receptor-related protein (LRP) with a molecular mass of >500 kDa has recently
5 been identified and characterized as a new member of the LDL receptor family (Herz, J. et al., (1988) EMBO J. 7, 4119-4127; Beisiegel, U. et al., (1989) Nature 341, 162-164; Brown, M.S. et al., (1991) Curr. Opin. Lipidol. 2, 65-72). This plasma membrane receptor is unique not only because of its large
10 molecular size but also because of its multifunctional nature in ligand recognition. Studies have shown that LRP mediates binding and endocytosis of both apoprotein E-enriched β -migrating very low density lipoprotein (β -VLDL) (Beisiegel, U. et al., (1989) Nature 341, 162-164; Lund, H. et al., (1989)
15 Proc. Natl. Acad. Sci. USA 86, 9318-9322) and protease- or methylamine-activated α_2 -macroglobulin (α_2 M) (Strickland, D.K. et al., (1990) J. Biol. Chem. 265, 17401-17404). Based on the following cross-linking studies, it was found that LRP functions as a MH₁C₁ cell specific 39kDa protein receptor.

20 Metabolic Labelling

Cells were grown in 10 cm dishes as described above in Example 3. Cell monolayers were washed three times with pre-warmed Eagle's minimum essential media (MEM) with Earle's salts lacking L-methionine (Washington University Center for
25 Basic Cancer Research). Following two 15 minute pre-incubations at 37°C with this same media, cells were labelled by the addition of methionine deficient MEM containing 0.4 mCi/ml ³⁵S-methionine (Amersham Corp.).

Labelling was carried out for 4-5 hours at 37°C. After metabolic labelling, cell monolayers were cooled on ice, washed three times with cold PBSc, and incubated with 14 nM of the radiolabelled ¹²⁵I-recombinant 39kDa protein of Example 3, either in the presence or absence of an excess of unlabelled 39kDa protein, to allow specific binding to the cell surface receptor.

Chemical Cross-Linking

Saturation binding was performed as described above, but with 10 cm dishes of MH₁C₁ cells. Either unlabelled or ³⁵S-methionine labelled cells were used. Following incubation with radiolabelled and unlabelled ligands (39kDa proteins), cell monolayers were washed three times with PBSc to remove non-specifically associated ligand and incubated with either PBSc alone or PBSc containing 0.5-1 mM dithiobis(sulfosuccinimidylpropionate) (DTSSP) (Pierce Chemical Co.), a disulfide bond containing, thiocleavable cross-linker. After one hour, cross-linking reactions were quenched by three washes with tris-buffered saline, and cell monolayers were solubilized in PBSa containing 1% Triton X-100 and 1 mM PMSF. Immunoprecipitations were performed as described below.

Immunoprecipitation

Aliquots of cell lysates from cross-linking and metabolic labelling experiments were added to equal volumes of PBSc containing 1% Triton X-100, 0.5% sodium deoxycholate, 1% sodium dodecyl sulfate (SDS), 0.5% bovine serum albumin, and 1mM PMSF (immunomix). When metabolically labelled cells were

used, lysates were first pre-cleared by an overnight incubation with rabbit pre-immune serum and a subsequent incubation with an excess of protein-A beads (Repligen). Samples were then immunoprecipitated with 0.6 μ g of affinity
5 purified α -39kDa antibody or affinity purified rabbit α -human LRP antibody (American Red Cross, Rockville, MD). Affinity purified α -39kDa antibody was prepared in New Zealand white rabbits following injection of purified recombinant 39kDa protein in accordance with the procedure described in
10 Schwartz, A.L. et al., (1983) J. Biol. Chem. 258, 11249-11255.

When unlabelled cells were cross-linked to a single radiolabelled ligand, no pre-immune clearance of cell lysates was necessary, and either α -LRP immune serum or α -39kDa protein immune serum was substituted for the affinity purified
15 antibody. In some cases, samples of cell lysates were also immunoprecipitated with 30 ml of immune α -t-PA serum. Following each immunoprecipitation, bead samples were split in half and boiled in 0.0625 M Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol as described in Laemmli, U.K., (1970) Nature 227,
20 680-685, either in the presence or absence of 5% 2-mercaptoethanol. Samples were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), with the gels subsequently fixed and vacuum dried. Autoradiography was performed at -70°C with Hyperfilm-MP
25 autoradiography film (Amersham Corp.).

FIG. 3 shows the results from a typical experiment. Under conditions where no cross-linker was used (FIG. 3C and D), a single band corresponding to the 39kDa protein

(arrowhead) was immunoprecipitated only with α -39kDa serum. Samples electrophoresed under either reducing or non-reducing conditions show that the α -LRP antibody was unable to immunoprecipitate the radiolabelled 39kDa ligand in the absence of cross-linker. Since there was a marked decrease in the amount of ^{125}I -39kDa protein bound in the presence of an excess of unlabelled protein, the association of ligand with its cell surface receptor was shown to be specific. In FIG. 3A and 3B, the radiolabelled 39kDa protein was both bound and cross-linked to the MH_1C_1 cell surface. When samples from each immunoprecipitation were analyzed under non-reducing conditions (FIG. 3A), the complex of ligand associated radioactivity was immunoprecipitable with both α -LRP and α -39kDa antibodies. This complex was of very high apparent molecular weight, remaining largely in the stacking gel during electrophoresis (arrows). Again, this interaction was shown to be specific, as the association of ^{125}I -39kDa with this high molecular weight complex was abrogated in the presence of an excess of unlabelled 39kDa protein. When these same samples were electrophoresed in the presence of 5% 2-mercaptoethanol to dissociate the cross-linker (FIG. 3B), a single 39kDa binding species was seen.

The foregoing experiments demonstrate that there is a specific interaction between the 39kDa protein and the MH_1C_1 cell surface which is mediated, at least in part, by an association with LRP.

EXAMPLE 6**Chemical Cross-linking of t-PA:PAI-1
Complex to LRP on HepG2 Cells**

Studies by Orth, K. et al., (1992) Proc. Natl. Acad. Sci. USA 89, 7422-7426, demonstrated via ligand blotting the binding of t-PA:PAI-1 complexes to isolated LRP immobilized on gels. Endocytosis by LRP and subsequent degradation of t-PA:PAI-1 complexes was also observed in simian COS tumor cells, a non-hepatocyte cell line. The following experiment showed that a direct interaction between the t-PA:PAI-1 complex and LRP in human hepatocytes occurs based on chemical cross-linking following ligand binding to human hepatoma HepG2 cells. In accordance with the present invention, it was hypothesized that binding and subsequent cross-linking of the t-PA:PAI-1 complex to LRP on HepG2 cells should yield a trimeric complex, t-PA:PAI-1-LRP. Therefore, if one component in this trimeric complex is radiolabelled, antibodies to the other components should be able to immunoprecipitate the radiolabelled protein. A thio-cleavable, water-soluble, and membrane-impermeant reagent DTSSP was used as the cross-linker, as described in Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602; and Bu, G. et al., (1992) Proc. Natl. Acad. Sci. USA 89, 7427-7431. Following ligand binding and cross-linking, cell lysates were used for immunoprecipitation with various antibodies, in order to characterize the nature of the cross-linked protein complexes. Chemical cross-linking experiments were performed with either ¹²⁵I-t-PA cross-linked to unlabelled HepG2 cells, or unlabelled t-PA cross-linked to ³⁵S-methionine metabolically-labelled HepG2 cells. Throughout

this example, protein concentration was determined using a Bio-Rad protein assay with bovine serum albumin as a standard. Cell number was determined by counting cells with a hemocytometer.

5 Metabolic Labelling

Cells growing in 10 cm dishes at about 80% confluence were incubated for 30 min at 37°C in two changes of Earle's minimum essential medium lacking L-methionine and containing 2 mM L-glutamine (Gibco Laboratories). Metabolic labelling was initiated by the addition of the above medium supplemented with ³⁵S-methionine (400 µCi/ml) (Amersham). Following incubation for 5 hours at 37°C, cell monolayers were washed with binding buffer and were used for ligand binding and chemical cross-linking experiments as described below.

15 Chemical Cross-linking

Experiments were performed with either ¹²⁵I-labelled ligand cross-linked to unlabelled cells or unlabelled ligand cross-linked to ³⁵S-methionine metabolically-labelled cells. After ligand binding at 4°C, each cell monolayer was washed three times with PBSc. Chemical cross-linking was performed by incubating the cell monolayer with PBSc containing 0.5 mM dithiobis(sulfosuccinimidylpropionate) (DTSSP) (Pierce Chemical Co.). After 30 min at 4°C, the reaction was quenched by washing the cell monolayer two times with Tris-buffered saline (TBS). Cells were then solubilized in PBSc containing 1% (v/v) Triton X-100 (Sigma Chemical Co.) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co.) ("lysis buffer") for 30 min at 4°C with a brief sonication at

low power output and occasional vortexing. These solubilized cell lysates were subsequently used for immunoprecipitation.

Antibodies

All antibodies used in these studies were rabbit polyclonal antibodies. Anti-t-PA, anti-PAI-1, and anti-39kDa protein antibodies were produced using recombinant proteins. Anti-LRP antibody was generated against purified human placental LRP provided by Dudley K. Strickland (American Red Cross, Rockville, Maryland). Total IgG for each antibody was purified using Protein A-agarose. Specific IgG for each antibody was affinity-purified against its corresponding protein coupled to CNBr-activated Sepharose 4B (Pharmacia).

Immunoprecipitation

Each cell lysate from chemical cross-linking experiments was divided into equal portions depending upon the number of antibodies used. Each aliquot of the cell lysate was then brought to 0.5 ml with lysis buffer and was mixed with 0.5 ml of PBSc containing 1% (v/v) Triton X-100 (Sigma Chemical Co.), 0.5% (w/v) sodium deoxycholate (Sigma Chemical Co.), 1% (w/v) SDS, 0.5% (w/v) bovine serum albumin (Sigma Chemical Co.), and 1 mM PMSF (Sigma Chemical Co.) ("immunomix"). SDS was included in the immunomix to reduce non-specific immunoprecipitation. Primary antibody (10 μ l normal rabbit serum (Sigma Chemical Co.), or 1 μ g affinity-purified IgG) was added, and the samples were rocked overnight at 4°C followed by incubation for 1 hour at room temperature with 50 μ l of protein A-agarose (Repligen). Non-bound radioactivity was removed by washing protein A-agarose beads

three times with immunomix and three times with PBSc. The protein A-agarose beads were divided into two equal parts before the final wash for nonreducing and reducing assays. The immunoprecipitated material was then released from the beads by boiling each sample for 5 min in 0.0625 M Tris-HCl, pH 6.8, 2% (w/v) SDS, and 10% (v/v) glycerol ("Laemmli sample buffer") (Laemmli, U. K., (1970) Nature 227, 680-685) with or without 5% (v/v) 2-mercaptoethanol, and was analyzed by SDS-PAGE as described below.

10 SDS-PAGE and Autoradiography

Samples of cell lysates or immunoprecipitations were analyzed by SDS-PAGE using polyacrylamide slab gels as described in Laemmli, U. K., (1970) Nature 227, 680-685, under reducing or non-reducing conditions. The following pre-stained molecular weight standards from Bio-Rad were used: myosin: 205 kDa; β -galactosidase: 117 kDa; bovine serum albumin: 80 kDa; ovalbumin: 50 kDa. Autoradiography of ^{125}I -labelled proteins was performed with dried polyacrylamide gels using Hyperfilm-MP (Amersham). For fluorography of ^{35}S -labelled proteins, gels were impregnated with Amplify (Amersham), dried, and exposed to films. Films were placed at -70°C for various periods of time as specified in each figure prior to developing.

FIG. 4 shows an experiment with ^{125}I -t-PA binding and cross-linking to HepG2 cells. Cell lysates without or with cross-linking were immunoprecipitated with one of the following antibodies: normal rabbit serum, anti-t-PA antibody, anti-PAI-1 antibody, or anti-LRP antibody. The

immunoprecipitated material was then analyzed by SDS-PAGE under nonreducing (FIG. 4A) or reducing (FIG. 4B) conditions. Under nonreducing conditions and without cross-linking, the ^{125}I -t-PA:PAI-1 complex was immunoprecipitated by both anti-t-PA (lane 2) and anti-PAI-1 antibody (lane 3) confirming the PAI-1-dependent ^{125}I -t-PA binding to HepG2 cells with the ^{125}I -t-PA:PAI-1 complex being the vast majority of the bound ligand. Uncomplexed ^{125}I -t-PA was observed with anti-t-PA antibody (lane 2) but not with anti-PAI-1 antibody (lane 3).

10 The appearance of the uncomplexed ^{125}I -t-PA (about 40% of the total cell-associated ligand radioactivity) in post-binding lysates was predominantly due to dissociation of ^{125}I -t-PA and PAI-1 during immunoprecipitation and washing with the immunomix containing 1% SDS and 1% Triton X-100. When the

15 cell lysates were analyzed directly using low-SDS sample buffer without prior immunoprecipitation, more than 90% of the specific binding species was in the form of ^{125}I -t-PA:PAI-1 complex as appears in FIG. 5B (data not shown). In the absence of cross-linking, anti-LRP antibody did not

20 immunoprecipitate any ^{125}I -labelled ligand (lane 4) indicating that this antibody does not have any cross-reactivity with the ^{125}I -t-PA:PAI-1 complex. However, with chemical cross-linking, anti-LRP antibody (lane 8), in addition to anti-t-PA antibody (lane 6) and anti-PAI-1 antibody (lane 7), immunoprecipitated

25 ^{125}I -labelled ligands. Since anti-LRP antibody does not cross-react with the ^{125}I -t-PA:PAI-1 complex (lane 4), this specific immunoprecipitation by anti-LRP antibody must be a result of the ^{125}I -t-PA:PAI-1 complexes cross-linked to LRP. The ligand-

receptor complexes consisting of ^{125}I -t-PA:PAI-1 and LRP appeared as a broad smear migrating on top of a 7% SDS-polyacrylamide gel with a molecular mass estimated to be greater than 600 kDa. However, when the same material was analyzed by SDS-PAGE under reducing conditions, the radioligand ^{125}I -t-PA:PAI-1 complex and ^{125}I -t-PA appeared following reduction of the thiocleavable cross-linker DTSSP (FIG. 4B). Reduction of the cross-linked material caused additional dissociation of the ^{125}I -t-PA:PAI-1 complex resulting in an even greater amount of free ^{125}I -t-PA. Normal rabbit serum showed no specific interaction with any of the cross-linked material (lanes 1 and 5).

To exclude the possibility that chemical cross-linking of ^{125}I -t-PA:PAI-1 to HepG2 cells generated an antigenic epitope for anti-LRP antibody, unlabelled t-PA was cross-linked to ^{35}S -methionine metabolically-labelled HepG2 cells. This approach allowed direct visualization of the binding protein for the t-PA:PAI-1 complex or the 39kDa protein and the determination of its relationship to LRP. Following ^{35}S -methionine metabolic labeling of HepG2 cells, cell monolayers were incubated with binding buffer alone, or binding buffer containing t-PA (15 nM) or the 39kDa protein (15 nM) (FIG. 6). Cell monolayers in the absence of ligand was lysed directly without chemical cross-linking whereas those following ligand binding were subjected to chemical cross-linking with DTSSP. Each of the cell lysate preparations was then immunoprecipitated with one of the following antibodies: normal rabbit serum (lanes 1, 6, 11),

anti-t-PA antibody (lanes 2, 7, 12), anti-PAI-1 antibody (lanes 3, 8, 13), anti-39kDa protein antibody (lanes 4, 9, 14), or anti-LRP antibody (lanes 5, 10, 15). The immunoprecipitated materials were then analyzed by 7% SDS-PAGE under either nonreducing or reducing conditions.

As shown in FIG. 6, anti-PAI-1 antibody immunoprecipitated PAI-1 from all the HepG2 cell lysates consistent with the well recognized production of PAI-1 by HepG2 hepatoma cells. (See, Bugelski, P. J. et al., (1989) *Thromb. Res.* 53, 287-303; Owensby, D. A. et al., (1991) *J. Biol. Chem.* 266, 4334-4340; Bu, G. et al., (1992) *J. Biol. Chem.* 267, 15595-15602; Sprengers, E. D. et al., (1985) *J. Lab Clin. Med.* 105, 751-758). When an ³⁵S-methionine-labelled HepG2 monolayer was analyzed without incubation with either t-PA or 39kDa protein, only the anti-LRP antibody was able to immunoprecipitate the two LRP subunits (515kDa and 85kDa) (lanes 5). However, following binding of unlabelled t-PA and chemical cross-linking, the ³⁵S-labelled ligand-receptor complexes were immunoprecipitable not only by anti-LRP antibody (lanes 10) but also by anti-t-PA antibody (lanes 7) and anti-PAI-1 antibody (lanes 8). When these materials were analyzed under reducing conditions, the LRP subunits, as well as the t-PA:PAI-1 complexes were observed. These results are thus consistent with the nature of the high molecular weight receptor-ligand complexes composed of the t-PA:PAI-1 complex (ligand) and LRP (receptor). Radiolabelling of the t-PA:PAI-1 complex was a result of complex formation between unlabelled t-PA and endogenous ³⁵S-methionine-labelled PAI-1.

When the ^{35}S -methionine-labelled HepG2 cell monolayer was exposed to externally added 39kDa protein, the ^{35}S -methionine-labelled ligand-receptor complex appearing on top of the gel was immunoprecipitable not only by anti-LRP antibody (lanes 15), but also by anti-39kDa protein antibody (lanes 14). Analysis of these cross-linked materials under reducing conditions yielded the 515kDa radiolabelled LRP band when immunoprecipitated with anti-39kDa antibody, further demonstrating the receptor protein for 39kDa protein on HepG2 cells is LRP.

EXAMPLE 7

The 39kDa Protein is Cross-linked Predominately to LRP on the MH_1C_1 Cell Surface

It was found that LRP is the predominant 39kDa protein binding species by cross-linking unlabelled 39kDa protein to the surface of radiolabelled MH_1C_1 cells using the procedures of Example 5 with the following modifications. Labelling was carried out at 37°C for 4-5 hours in methionine deficient Earle's MEM media supplemented to 0.4 mCi/ml with ^{35}S -methionine (Amersham Corp.). Following several washes, labelled monolayers were bound with unlabelled 39kDa protein, and either cross-linked by the addition of 0.5 mM DTSSP, or incubated in PBSc alone. Lysis and immunoprecipitation were carried out as described above in Example 5.

FIG. 7 shows the results from a typical experiment. When radiolabelled MH_1C_1 cells were cross-linked to unlabelled 39kDa protein, a complex of very high apparent molecular weight resulted, which was immunoprecipitable with both the α -39kDa and, to a lesser degree, the α -human LRP affinity

purified antibodies (FIG. 7A, lanes 4-6). Antibodies to human LRP were used as no α -rat LRP affinity purified antibody is presently available. This high molecular weight complex was electrophoretically identical to that seen when radiolabelled ¹²⁵I-39kDa protein was cross-linked to unlabelled MH₁C₁ cells (compare with FIG. 3A, lanes 2 and 3). In addition to the cross-linked complex, uncross-linked endogenously labelled 39kDa protein (lane 5, arrowhead), and both the 520kDa (closed arrow) and 85kDa (open arrow) subunits of the LRP receptor were visible. When these same immunoprecipitations were electrophoresed in the presence of the reducing agent 2-mercaptoethanol, the cross-linker was cleaved, and the cross-linked complex was dissociated into its component peptides (FIG. 7B, lanes 4-6). In both cases, the cross-linked complex was shown to contain almost exclusively the 520kDa subunit, and to a lesser degree, the 85kDa subunit of the LRP receptor. In lanes resulting from immunoprecipitation with the α -human LRP antibody, less of the high molecular weight cross-linked complex was evident. It is believed that this occurs partly because the recognition epitopes for the α -LRP antibody are obscured by binding of the 39kDa protein.

When metabolically labelled cell lysates which have been bound with, but not cross-linked to, unlabelled 39kDa protein were immunoprecipitated with the α -39kDa antibody, only endogenously labelled 39kDa protein was seen (arrowhead). No high molecular weight complex was evident. Also, in the absence of cross-linker, immunoprecipitation of metabolically

labelled cell lysates with α -human LRP antibodies results in only the 520kDa (closed arrow) and the 85kDa (open arrow) subunits of the LRP receptor. Again, no high molecular weight complex was evident. Samples electrophoresed under both
5 reducing and non-reducing conditions gave similar results.

This example demonstrates that the LRP is the predominant 39kDa protein binding species on the MH_1C_1 cell surface.

* * * * *

10 Example 8 demonstrates that both the 39kDa protein and t-PA specifically co-bind to a single LRP molecule.

EXAMPLE 8

t-PA and the 39kDa Protein can be Cross-linked to
the Same LRP Receptor Complex

15 0.5nM ^{125}I -39kDa protein and 20nM unlabelled t-PA were co-bound to MH_1C_1 cells both in the presence and absence of an excess of unlabelled 39kDa protein. Following several washes to remove non-specifically associated ligand, cell monolayers
20 were either incubated in 1mM DTSSP or in PBSc alone. Once cross-linked, cell monolayers were washed, lysed and immunoprecipitated as described above.

FIG. 8 shows the results from a typical experiment. When the ^{125}I -39kDa protein and unlabelled t-PA were co-bound to
25 the MH_1C_1 cell surface and incubated in the absence of cross-linker (FIG. 8C and D), the radiolabelled 39kDa protein was immunoprecipitated from cell lysates with only the α -39kDa antibody (arrowhead). Neither the α -LRP antibody nor the α -t-PA antibody showed any affinity for the radiolabelled
30 39kDa ligand. Samples electrophoresed under both reducing and

non-reducing conditions gave similar results. As in Example 3, interaction of the 39kDa protein with the MH₁C₁ cell surface was shown to be specific by abrogation with an excess of unlabelled 39kDa protein. When MH₁C₁ cells were pre-bound with ¹²⁵I-39kDa protein and unlabelled t-PA, and incubated in the presence of cross-linker (FIG. 8A and B), a high molecular weight complex of ligand-associated radioactivity resulted, which was immunoprecipitable with α-39kDa, α-t-PA, and α-LRP antibodies. Each of these interactions was specific, as each was abolished in samples incubated in the presence of an excess of unlabelled 39kDa protein. This protein complex of ¹²⁵I-39kDa, t-PA, and LRP was of very high apparent molecular weight as before, remaining largely in the stacking gel during electrophoresis (arrows). When these same samples were electrophoresed in the presence of 2-mercaptoethanol to cleave the cross-linker, each resulted in a single radiolabelled band corresponding to the exogenously added ¹²⁵I-39kDa protein (arrowhead).

In order to determine if cross-linking of the 39kDa protein to the MH₁C₁ cell surface generates an epitope for recognition by the α-t-PA antibody, similar experiments were performed with ¹²⁵I-39kDa protein bound and cross-linked to the MH₁C₁ cell surface, but in the absence of exogenously added, unlabelled t-PA (FIG. 8E and F). In this case, the high molecular weight complex of ligand-associated radioactivity was immunoprecipitable only with α-LRP and α-39kDa antibodies, and not with the α-t-PA antibody.

This experiment shows that the simple cross-linking of radiolabelled 39kDa protein to the MH_1C_1 cell surface does not change its specificity for either the α -t-PA or α -39kDa antibodies. A number of additional co-binding and cross-linking experiments, performed using ^{125}I -t-PA and unlabelled 39kDa protein, gave similar results (data not shown). This data taken together with the data presented above leads to the conclusion that the 39kDa protein and t-PA can bind simultaneously to LRP on the MH_1C_1 cell surface.

* * * * *

It was found from the following examples that endocytosis of the 39kDa protein is receptor-mediated like t-PA and that the 39kDa protein modulates the receptor-mediated endocytosis of t-PA.

EXAMPLE 9

Rapid Uptake and Degradation of ^{125}I -39kDa by MH_1C_1 Cells

It is known that MH_1C_1 cells rapidly endocytose and degrade t-PA (Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602). The following single cycle uptake and degradation experiments demonstrate that MH_1C_1 cells similarly endocytose the 39kDa protein.

Endocytosis experiments were performed according to Ciechanover et al., (1983) J. Biol. Chem. 258, 9681-9689 and Owensby et al., (1988) J. Biol. Chem. 263, 10587-10594. 6 well dishes containing 10^6 cells/well were preincubated at 4°C with 14 nM ^{125}I -39kDa protein to allow binding. Following three washes with PBSc (4°C) to remove non-specifically associated ligand, each dish was incubated at 37°C, and a pre-warmed

solution of 200 nM unlabelled 39kDa protein was added to initiate internalization. At each time point, over a period of 60 minutes, one of the dishes was cooled directly on ice, and the overlying media was removed. Precipitation of the overlying media was carried out by the addition of bovine serum albumin to 10 mg/ml and trichloroacetic acid (TCA) to 20%. Both the TCA soluble (i.e., extracellular degraded ligand) and TCA insoluble (i.e., extracellular dissociated ligand) fractions were counted by γ scintillation spectrometry.

In addition, cell monolayers were washed three times with PBSc and incubated 30 minutes at 4°C with a solution of PBSc containing 0.25% Pronase (Calbiochem) to digest membrane-associated ligand. Cells were subsequently removed from each well and pelleted by centrifugation at 12,000 g. Both the cell pellets and the supernatants following Pronase treatment were counted by γ scintillation spectrometry to determine both cell associated (i.e., protease resistant) and membrane-associated (i.e., protease sensitive) reactivity.

FIG. 9 shows the results from a typical experiment. As expected, all ligand-associated radioactivity was initially located at the cell surface. Within 3 minutes of warm-up, however, 80% of this population became intracellular (protease resistant) or dissociated into the media as TCA insoluble (undegraded ligand) counts. The intracellular levels of ligand reached a peak value of 50% of total at approximately 10 minutes. Concurrently, the TCA soluble radioactivity, representing internalized and subsequently degraded ligand,

appeared in the overlying media initially at 10 minutes, with plateau levels reached at between 45 and 60 minutes. This kinetic pattern of uptake and degradation of a single cohort of pre-bound ^{125}I -39kDa ligand was identical to that observed for ^{125}I -t-PA with these MH_1C_1 cells.

EXAMPLE 10

Inhibition of ^{125}I -t-PA Uptake and Degradation by the 39kDa Protein on HepG2 Cells

The receptor-mediated endocytosis of the t-PA:PAI-1 complex by HepG2 cells has been previously demonstrated. (See, Owensby, D. A. et al., (1988) J. Biol. Chem. 263, 10587-10594; Morton, P. A. et al., (1989) J. Biol. Chem. 264, 7228-7235; Underhill, D. M. et al., (1992) Blood 80, 2746-2754). It was found from the following experiment that the endocytosis and degradation of the t-PA:PAI-1 complex by HepG2 cells were also via LRP and that the 39kDa protein inhibits the endocytosis and degradation of ^{125}I -t-PA.

Throughout this example, protein concentration was determined using a Bio-Rad protein assay with bovine serum albumin as a standard. Cell number was determined by counting cells with a hemocytometer.

Ligand Uptake and Degradation

HepG2 cell monolayers seeded in 6-well dishes were incubated at 37°C with 3 nM ^{125}I -t-PA in the absence or presence of various competitors at selected concentrations. After selected intervals, binding buffer overlying cell monolayers was collected, cell monolayers were chilled on ice and washed with prechilled binding buffer for three times to prevent further ligand uptake and degradation. Cellular degradation

of radiolabelled ligand was determined by measuring the appearance of TCA-soluble radioactivity in the overlying buffer, whereas cell-associated radioactivity was determined by lysing the cell monolayers in the low-SDS lysis buffer and measuring the total radioactivity in the cell lysates. Cell-mediated degradation of ^{125}I -t-PA was monitored in the absence or presence of an excess of one of the following competitor proteins: t-PA, 39kDa protein, or BSA.

The results shown in FIG. 10A demonstrate that, in the absence of any competitor protein, the degradation of ^{125}I -t-PA (initial concentration=3 nM) increased almost linearly for at least 4 hours. This observation suggests the existence of an intracellular receptor pool and recycling of the t-PA receptors. (See, Owensby, D. A. et al., (1988) J. Biol. Chem. 263, 10587-10594; Morton, P. A. et al., (1989) J. Biol. Chem. 264, 7228-7235). Excess unlabelled t-PA (1 μM) reduced the degradation of ^{125}I -t-PA to about 10% which represents the nonspecific degradation of the ligand. When the 39kDa protein was included in the incubation, specific degradation of ^{125}I -t-PA was inhibited by about 75%. This is consistent with the majority of the specific degradation occurring via LRP. This degree of inhibition of ^{125}I -t-PA degradation by the 39kDa protein is similar to the degree of inhibition of ^{125}I -t-PA binding by the 39kDa protein (see FIG. 5). The control protein BSA had no effect on ^{125}I -t-PA degradation.

To confirm that the inhibition of ^{125}I -t-PA degradation by the 39kDa protein occurs at the step of ligand uptake rather than intracellular degradation, the cell-

associated radioactivity was quantitated during the first hour of endocytosis. As shown in FIG. 10B, about 70% of the specific ^{125}I -t-PA uptake was inhibited by the 39kDa protein, whereas the control protein BSA showed no inhibitory effect on this process.

These results clearly demonstrate that LRP serves as the major PAI-1-dependent endocytosis receptor for t-PA on HepG2 cells, and that the 39kDa LRP receptor-associated protein modulates this interaction.

* * * * *

MH₁C₁ cells rapidly catabolize the 39kDa protein as shown in Example 11.

EXAMPLE 11

Rate of 39kDa Protein Uptake by MH₁C₁ Cells

The following experiment used the procedure described in Schwartz, A.L. et al., (1982) J. Biol. Chem. 257, 4230-4237.

Rate of 39kDa Protein Uptake

Dishes containing monolayers of 10^6 MH₁C₁ cells/well were cooled on ice and washed three times with cold PBSc. Ligand uptake was initiated by the addition of prewarmed (37°C) binding buffer containing 28 nM ^{125}I -39kDa protein, either in the presence or absence of an excess of unlabelled 39kDa protein. Each dish was incubated at 37°C for a period of between 0 and 120 minutes. At each time point, one of the dishes was cooled directly on ice, and the overlying media was removed. Cell monolayers were washed three times with PBSc and lysed in low SDS lysis buffer for the determination of

cell associated radioactivity. Each sample of overlying media was precipitated with TCA as described in Example 9, and the TCA soluble fraction was counted by γ scintillation spectrometry.

5 FIG. 11 shows the results from a typical experiment. The amount of cell-associated ligand increased rapidly, reaching a plateau at approximately 40 minutes after the transition in temperature. The amount of extracellular degraded ligand exhibited a lag of approximately 20 minutes,
10 after which there began a linear increase which lasted the duration of the experiment. The sum of both cell-associated and extracellular degraded ligand also increased linearly with a slope identical to that of extracellular degraded ligand alone. The slopes of each of these parameters was a measure
15 of the rate of ligand uptake and degradation at 37°C and was determined to be 52 fmol/10⁶ cells/minute. This experiment demonstrates a pattern of rapid catabolism of the 39kDa protein by MH₁C₁ cells.

* * * * *

20 The following examples demonstrate that the 39kDa protein inhibits t-PA binding to hepatic receptors.

EXAMPLE 12

Rat 39kDa Protein Can Efficiently Inhibit t-PA Binding to MH₁C₁ Cells

25 Using the procedure described in Example 3, the ¹²⁵I-t-PA binding analyses described below showed the kinetics of inhibition of t-PA binding to MH₁C₁ cells by the native rat 39kDa protein. FIG. 12 shows the results of ¹²⁵I-t-PA binding analyses performed in the presence of varying concentrations

of either unlabelled t-PA or recombinant rat 39kDa protein as competitors. Unlabelled t-PA inhibits binding of ^{125}I -t-PA with an apparent K_i value of 8 nM (FIG. 12B). The 39kDa protein, on the other hand, competed at a much lower concentration, with an apparent K_i value of 0.5 nM (FIG. 12A). This experiment demonstrates that the 39kDa protein is a strong competitor for ^{125}I -t-PA binding to LRP on the MH_1C_1 cell surface.

EXAMPLE 13

Inhibition of ^{125}I -t-PA:PAI-1 Complex Binding to HepG2 Cells by 39kDa Protein and Anti-LRP Antibody

In the following ligand binding competition experiments, it was found that PAI-1-dependent ^{125}I -t-PA binding was also mediated via LRP. Specific binding of 3 nM ^{125}I -t-PA was performed in the absence or presence of each of three competitor proteins: t-PA, 39kDa protein, or bovine serum albumin (BSA). Each competitor protein was included at increasing concentrations up to more than 100-fold molar excess of the radiolabelled ligand. Throughout this example, protein concentration was determined using a Bio-Rad protein assay with bovine serum albumin as a standard. Cell number was determined by counting cells with a hemocytometer.

Ligand binding competition assays

Binding of the ^{125}I -labelled ligand was performed as described above in Example 4 in the absence or presence of various competitors at selected concentrations. In those experiments in which both t-PA and 39kDa protein were involved, the t-PA binding buffer was used. Nonspecific binding was determined in the presence of 1 μM unlabelled ligand.

As shown in FIG. 5A, unlabelled t-PA competed with ^{125}I -t-PA for binding with an apparent K_i of about 3 nM. The 39kDa protein also inhibited specific ^{125}I -t-PA binding to a maximum of about 80% (FIG. 5A). The control protein BSA had no effect on specific ^{125}I -t-PA binding indicating that inhibition by the 39kDa protein was not due to a generalized effect but was specific. These results with the 39kDa protein suggest that the majority of the PAI-1-dependent ^{125}I -t-PA binding on the HepG2 cells is via LRP. The observation that the 39kDa protein inhibits only 80% of specific ^{125}I -t-PA binding may indicate the presence of a minor population of t-PA binding sites that are not accessible to or inhibited by the 39kDa protein.

To test whether inhibition of ^{125}I -t-PA binding by the 39kDa protein was due to interference with formation of the complex between ^{125}I -t-PA and endogenous PAI-1, post-binding buffer overlying cell monolayers from the binding assays of FIG. 5A (without any competitor protein or from each of those with 500 nM competitor protein) was analyzed by SDS-PAGE (FIG. 5B). The ^{125}I -t-PA:PAI-1 complex can be seen in the absence of excess unlabelled t-PA (lane 1), but not in the presence of excess unlabelled t-PA (lane 2). Inclusion of excess unlabelled 39kDa protein did not interfere with the formation of the ^{125}I -t-PA:PAI-1 complex (lane 3). The ^{125}I -t-PA:PAI-1 complex observed in the presence of the 39kDa protein is greater than that seen with ^{125}I -t-PA alone and presumably reflects the inhibition of complex binding to HepG2 cells by the 39kDa protein. Cell lysates corresponding to the

overlying buffer shown in lane 1-4 were also analyzed via the same SDS polyacrylamide gel (FIG. 5B). Binding of the ^{125}I -t-PA:PAI-1 complex to HepG2 cells is demonstrated in the cell lysates without competitor protein (lane 5). When excess unlabelled t-PA was included, however, specific binding of the ^{125}I -t-PA:PAI-1 complex was absent (lane 6). When the cell lysate with 500 nM 39kDa protein was compared to that with ^{125}I -t-PA alone, specific ^{125}I -t-PA:PAI-1 complex binding was reduced by about 80% (lane 7). The control protein BSA neither interfered with the complex formation (lane 4) nor inhibited specific ^{125}I -t-PA:PAI-1 complex binding (lane 8). These results thus demonstrate that the inhibition of ^{125}I -t-PA binding to HepG2 cells by the 39kDa protein occurs at the level of ^{125}I -t-PA:PAI-1 binding to LRP without interference with the initial complex formation.

Modulation of ligand binding to LRP by the 39kDa protein is not due to competition at a single site with various ligands since previous experiments have shown that LRP ligands (e.g., $\alpha_2\text{M}$, t-PA) compete only slightly with the 39kDa protein for binding to LRP. (See, Bu, G. et al., (1992) Proc. Natl. Acad. Sci. USA 89, 7427-7431). To test whether ^{125}I -39kDa protein binding is affected by t-PA on HepG2 cells, ligand binding competition experiments were performed. Competitor proteins (t-PA, 39kDa protein, or BSA) at various concentrations were included in ^{125}I -39kDa protein binding. As shown in FIG. 13, specific binding of ^{125}I -39kDa protein was reduced only slightly (about 20%) by excess unlabelled t-PA, whereas the binding was completely inhibited by excess

unlabelled 39kDa protein. These results suggest that the 39kDa protein inhibits t-PA:PAI-1 complex binding indirectly perhaps via inducing a conformational change in LRP unfavorable to t-PA:PAI-1 complex binding. Alternatively, 5 t-PA:PAI-1 complex binding sites on HepG2 cells may account for only a small portion of the 39kDa protein binding sites with most of the binding sites distributed over other parts of the LRP molecule not shared with t-PA.

To test directly whether the majority of PAI-1- 10 dependent ^{125}I -t-PA binding sites is on LRP, ligand binding competition experiments were performed in the presence of anti-LRP antibody. This rabbit polyclonal antibody was generated using purified human placental LRP as antigen. The total IgG fraction was purified and used for competition of 15 ^{125}I -t-PA binding (3 nM). Non-immune rabbit IgG was used as a control. As shown in FIG. 14, specific binding of the ^{125}I -t-PA:PAI-1 complex was reduced by up to 75%, while non-immune IgG had little or no effect. The percentage of reduction on specific ^{125}I -t-PA binding by anti-LRP antibody (i.e. 20 approximately 75%) is similar to that seen with the 39kDa protein (see FIG. 5), further supporting the observation that the predominant binding (~70-80%) of the ^{125}I -t-PA:PAI-1 complex on HepG2 cells is mediated by LRP.

* * * * *

25 In the following example, it was found that the 39kDa protein effectively inhibited hepatic clearance of t-PA in both *in vitro* and *in vivo* experiments.

EXAMPLE 14**Effect of 39kDa Protein on Hepatic Clearance of t-PA**39kDa Protein Preparation

The 39kDa protein was isolated from 5 liters of *E. coli* containing the recombinant plasmid according to Example 1 and yielded the GST-39kDa protein. Following thrombin cleavage and removal of the GST, the 39kDa protein was approximately 95% pure as determined by SDS-PAGE and Coomassie/silver staining. The few percent of non-39kDa protein contained within these preparations were unabsorbed fragments of GST. In order to provide a homogeneously pure preparation of 39kDa protein, this partially purified mixture was applied to a heparin agarose column (Sigma Chemical Co.). Following extensive washing with 100 mM NaCl to remove contaminating proteins, the 39kDa protein was eluted at 0.4 M NaCl. This preparation resulted in the greater than 99% homogenous preparation of 39kDa protein as determined by SDS-PAGE and Coomassie/silver staining and shown in FIG. 15.

General Procedures

The following procedures were used throughout this example.

1. Cell Culture

Rat hepatoma MH₁C₁ cells were cultured in accordance with the procedure of Example 3.

2. Protein Iodination

t-PA, 39kDa protein, GST, α 1-acid glycoprotein (orosomuroid, OR), and the asialo form of α 1-acid glycoprotein (ASOR) were iodinated with ¹²⁵I as described in Example 4. The

specific activity of the resultant labelled proteins was 5-10 $\mu\text{Ci}/\mu\text{g}$ of protein.

3. Protein Concentration Determination

Protein concentration was determined using the Biorad protein assay with bovine serum albumin as a standard.

Ligand Binding Assays

Bioactivity was determined on this preparation of 39kDa protein by its ability to inhibit ^{125}I -t-PA to MH_1C_1 cells as described in Example 9. Wells containing approximately 10^6 MH_1C_1 cells were washed and preincubated at 4°C with binding buffer containing the indicated concentrations of unlabelled t-PA or unlabelled 39kDa protein in addition to 3 nM ^{125}I -t-PA. Following incubation for 1.5 hours at 4°C , the medium was removed, the cells washed and the specific ^{125}I -t-PA binding was determined based on the radioactivity measured in accordance with the procedure of Example 4. As seen in FIG. 15, this preparation of 39kDa protein is biologically active against the t-PA receptor with 50% inhibition seen at <1 nM.

In vivo Clearance Determinations

In vivo clearance determinations were performed in the following manner on 200 gm female Sprague-Dawley rats obtained from Charles River Breeders and fed standard lab chow ad lib. Following anesthesia with 12 mg Nembutal, the tail vein was catheterized and a 250 μl solution containing the test protein in normal saline was infused over 30 seconds. Repetitive samples were obtained from the distal tail artery into cooled microtubes containing 2 μl heparin (1:1000). Routinely samples were collected at 30 seconds, 1, 2, 4, 6, 8

and 10 minutes. After the 10 minute peripheral sample was collected, a central blood sample was collected by an open cardiac puncture and the visceral organs (liver, kidney, spleen) were rapidly removed to ice. Aliquots of the liver and entire kidney and spleen were counted for radioactivity determination. Heparinized blood samples were centrifuged to separate plasma from blood cells and an aliquot of plasma, generally 25 μ l, was spotted onto Whatman 3M paper, dried, precipitated in 10% trichloroacetic acid and radioactivity determined in a Packard Gamma Spectrophotometer.

1. Clearance of t-PA

To demonstrate that t-PA is rapidly cleared from the blood plasma following intravenous administration, adult 200 g rats were anesthetized and administered 30 pmol 125 I-t-PA via venous injection as described above. Arterial blood samples were collected at the indicated times, separated and plasma radioactivity determined.

As seen in FIG. 16A, following intravenous administration to each of the eight rats of 30 pmol of 125 I-t-PA, the plasma 125 I-t-PA was rapidly cleared with a $t_{1/2}$ of approximately one minute. Less than 10% of the 125 I-t-PA remained in blood plasma at 10 minutes. Analysis of the liver at 10 minutes following the administration revealed approximately 90% of the initial plasma radioactivity was found in liver.

2. Effect of Excess t-PA on t-PA Clearance

Intravenous administration to three rats of 400-fold molar excess unlabelled t-PA (12 nmol) one minute prior to

administration of the identical dose of 30 pmol ^{125}I -t-PA resulted in a marked reduction in the plasma clearance of ^{125}I -t-PA as seen in FIG. 16A. The plasma half-life was approximately 9-10 minutes and approximately 40% of the ^{125}I -t-PA remained in the blood at 10 minutes. Analysis of the liver at 10 minutes revealed approximately 70% of the initial plasma radioactivity was found in liver.

3. Effect of 39kDa Protein on t-PA Clearance

Intravenous administration to four rats of 250 nmol of unlabelled 39kDa protein (8,000-fold molar excess) one minute prior to administration of the identical 30 pmol ^{125}I -t-PA also resulted in a marked reduction in the plasma clearance of ^{125}I -t-PA as seen in FIG. 16A. Plasma half-life was approximately 6 minutes. Approximately 30% of the ^{125}I -t-PA remained in the blood at 10 minutes. Analysis of the liver at 10 minutes revealed approximately 80% of the initial plasma radioactivity was found in liver.

4. Clearance of 39kDa Protein

A series of controlled clearance experiments were performed to determine the *in vivo* fate of the administered 39kDa protein. Adult 200 g rats were anesthetized and administered 30 pmol ^{125}I -39kDa protein via venous injection. Arterial blood samples were collected at the indicated times, separated, and plasma radioactivity determined as described above. As seen in FIG. 17, following intravenous administration to the rat of 30 pmol of ^{125}I -39kDa protein, the plasma ^{125}I -39kDa protein was rapidly cleared with a $t_{1/2}$ of approximately one minute.

The following table, Table 2, shows that the analysis of the liver, kidney and spleen at 10 minutes revealed approximately 70% of the initial radioactivity in liver, 7% in kidney and less than 2% in spleen. Following plasma clearance of ^{125}I -39kDa protein in the rat *in vivo* as described in FIG. 17, the liver, kidney and spleen were harvested at 10 minutes and the radioactivity determined. Each figure is the mean \pm S.E.M.

TABLE 2

ORGAN DISTRIBUTION OF ^{125}I -39kDa PROTEIN

Preadministration		^{125}I -39kDa Protein	Liver	Kidney	Spleen
(dose)		(dose)	(% initial radioactivity)		
a.	none	30 pmol	69 \pm 5	7 \pm 1	2 \pm 0.2
b.	12.5 nmol	30 pmol	72 \pm 3	12 \pm 1	2 \pm 0.2
c.	50 nmol	30 pmol	25 \pm 6	13 \pm 2	2 \pm 0.6
d.	125 nmol	30 pmol	23 \pm 5	31 \pm 10	2 \pm 0.3

The capacity for hepatic clearance of 39kDa protein is large, but can be saturated as seen in FIG. 17 and Table 2. Intravenous administration of increasing doses of unlabelled 39kDa protein results in a decrease of the fraction of the initial dose found in the liver at 10 minutes. Concomitantly there is an increasing fraction of the initial dose found in the kidney, which indicates that maximal hepatic capacity of 39kDa protein clearance can be readily achieved *in vivo*. Therefore multiple dosing schedules and/or continuous infusion of 39kDa protein should markedly reduce hepatic t-PA clearance *in vivo*.

5. Effect of Excess 39kDa Protein on 39kDa Protein Clearance

Intravenous administration of 12.5 nmol of unlabelled 39kDa protein one minute prior to administration of the identical 30 pmol of ^{125}I -39kDa protein resulted in a prolongation of the plasma half-life to approximately three minutes. Analysis of liver, kidney and spleen at 10 minutes revealed approximately 70% in liver, 12% in kidney, and less than 2% in spleen.

Intravenous administration of 50 nmol of unlabelled 39kDa protein one minute prior to the identical 30 pmol of ^{125}I -39kDa protein resulted in a further marked reduction in the plasma clearance of the ^{125}I -39kDa protein as seen in FIG. 17 with a half-life of approximately six minutes. Analysis of the liver, kidney and spleen at 10 minutes revealed approximately 30% liver, 15% in kidney and less than 2% in spleen.

Intravenous administration of 125 nmol of unlabelled 39kDa protein one minute prior to administration of the identical 30 pmol of ^{125}I -39kDa protein resulted in a further marked reduction in the plasma clearance of the ^{125}I -39kDa protein with a plasma half-life of approximately 9 minutes. Analysis of the liver, kidney and spleen revealed approximately 22% in liver, 33% in kidney and less than 2% in spleen.

6. Clearance of Other Proteins

Not all proteins administered to the rat intravenously are rapidly cleared by liver. Adult 200 g rats were anesthetized and administered via venous injection (a) 5

pmol ^{125}I - α 1-acid glycoprotein (OR), and (b) 5 pmol ^{125}I -asialo- α 1-acid glycoprotein (ASOR). Arterial blood samples were collected at the indicated times, separated, and plasma radioactivity determined as described above. As seen in FIG. 16B, for example, α 1-acid glycoprotein was cleared with a half-time of greater than 90 minutes, whereas its asialo-derivative was rapidly cleared with half-time of approximately 0.5 minute (see Schwartz et al., (1984) CRC Crit. Rev. Biochem. 16, 207-233).

EXAMPLE 15

Effect of 39kDa Protein Fragments on Hepatic Clearance of t-PA

The following experiment demonstrates that the entire 39kDa protein molecule is not required for inhibition of t-PA binding to the hepatic t-PA receptor. The 39kDa protein was chemically cleaved and the individual fragments isolated. These were then tested for inhibition of ^{125}I -t-PA binding to receptors on rat hepatocyte cells. Throughout this example, protein concentration was determined using a Bio-Rad protein assay with bovine serum albumin as a standard.

Purified 39kDa protein was prepared in accordance with the procedure of Example 14. In order to generate isolated fragments of the 39kDa protein, acid hydrolysis was performed under strict conditions. The 39kDa protein in 0.4M NaCl was incubated for 80 hours at room temperature in 70% formic acid. These acid proteolytic conditions provided for the complete cleavage of the intact 39kDa protein at the Asp-Pro bond at amino acid residues 114-115 and the generation of

two large fragments (~20kDa N-terminal fragment and ~28kDa C-terminal fragment).

Following separation on SDS-PAGE, the intact 39kDa protein, the 20kDa N-terminal fragment, and 28kDa C-terminal fragment were electrophoretically transferred to PVDF paper in accordance with the procedure described in Matsudaira, F., A Practical Guide to Protein and Peptide Purification for Microsequences (1989 Academic Press, NY). The appropriate polypeptide bands were visualized by staining representative gel lanes, cut out of the PVDF paper, and eluted by incubation for 1 hour at room temperature in 1% Triton X-100 in 50mM Tris, pH 9. Reanalysis by SDS-PAGE demonstrates single individual bands of the appropriate molecular weights for the intact 39kDa protein, the 20kDa N-terminal fragment, and the 28kDa C-terminal fragment. Sequencing of these individual protein species at the Washington University Microchemical Core Facility confirmed cleavage of the intact 39kDa protein at the Asp₁₁₄-Pro₁₁₅ bond.

Thereafter the biological activity of the intact 39kDa protein, the 20kDa N-terminal fragment, and the 28kDa C-terminal fragment were tested by quantitating the inhibition of ¹²⁵I-t-PA binding to rat liver MH₁C₁ cells. Cells were washed and preincubated at 4°C in binding buffer containing 0-16 nM of the intact 39kDa protein (●), the 20kDa N-terminal fragment (■), the 28kDa C-terminal fragment (▲), or PVDF-elution buffer alone (○) as a control. 4 nM ¹²⁵I-t-PA was added and the incubation continued for 2 hours at 4°C. Following removal and washing of the unbound ¹²⁵I-t-PA, the amount of

radioactivity bound to the MH_1C_1 cells was determined as described in Example 14.

As shown in FIG. 18, the intact 39kDa protein inhibited ^{125}I -t-PA binding with 50% inhibition at approximately 1 nM and 90% inhibition at approximately 8-10 nM, consistent with previous observations (Bu et al., (1992) Proc. Natl. Acad. Sci. USA 89, 7427-7431; Examples 10, 12, 13, and 14 above). The 20kDa N-terminal fragment was essentially without effect (i.e., approximately 10% inhibition at 16 nM; buffer alone exhibited 20-30% inhibition under these identical conditions). In marked contrast the 28kDa C-terminal fragment was an extremely active inhibitor of ^{125}I -t-PA binding with approximately 50% inhibition at 4 nM and 90% inhibition at 16 nM as shown in FIG. 18.

The nearly equipotent activity of the 28kDa C-terminal fragment compared to intact 39kDa protein strongly suggests that a small fragment of the 39kDa protein, which could include a fragment that overlaps the dividing point between the 20kDa N-terminal fragment and 28kDa C-terminal fragment, or a chemically or genetically related/modified form of the molecule, will be recognized by the t-PA hepatic receptor and may serve as a powerful reagent in inhibiting t-PA clearance *in vivo*.

* * * * *

The above description is meant to be illustrative of the present invention, and not limiting thereof. All explanations of the inventors' theory of the invention are for illustrative purposes only.

SEQUENCE LISTING

(1) GENERAL INFORMATION

- (i) APPLICANTS: Schwartz, Alan L., Bu, Guojun
- (ii) TITLE OF INVENTION: Methods And Compositions For Inhibition Of Hepatic Clearance Of Tissue-Type Plasminogen Activator
- (iii) NUMBER OF SEQUENCES: 5
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Bryan Cave
 - (B) STREET: 245 Park Avenue
 - (C) CITY: New York
 - (D) STATE: New York
 - (E) COUNTRY: USA
 - (F) ZIP: 10167-0034
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Diskette, 3.50 inch, double density 720 Kb storage
 - (B) COMPUTER: IBM PC Compatible
 - (C) OPERATING SYSTEM: DOS Version 3.3
 - (D) SOFTWARE: Word Perfect 5.1
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: To be assigned
 - (B) FILING DATE: 17-DEC-1993
 - (C) CLASSIFICATION: To be assigned
- (vii) PRIOR APPLICATION DATA
 - (A) U.S. APPLICATION NUMBER: 07/992,827
 - (B) U.S. FILING DATE: 18-DEC-1992
 - (C) U.S. CLASSIFICATION: Preliminary Class 514
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Barnhard, Elizabeth M.

(B) REGISTRATION NUMBER: 31,088

(C) REFERENCE/DOCKET NUMBER: 00108/064909

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (212) 692-1800

(B) TELEFAX: (212) 692-1900

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 323 amino acid residues

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

[illegible]

(3) INFORMATION FOR SEQ ID NO: 2:

(i) **SEQUENCE CHARACTERISTICS:**

(A) LENGTH: 209 amino acid residues

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

[illegible]

(4) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (oligonucleotide)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCGCGTGGAT CCCCAGGCT GGAAAAGCTG TGG

33

(5) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (oligonucleotide)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TCAATGAATT CTCAGAGTCG CTCGCCGTCG CCCAC

35

CLAIMS

1. A method of inhibiting the hepatic clearance of tissue-type plasminogen activator (t-PA) *in vivo* in humans comprising administering a t-PA-hepatic clearance-inhibiting amount of 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment thereof.

2. The method of claim 1, wherein the amount of 39kDa protein or fragment thereof ranges from about 60 to 6,000 mg/kg of body weight/dose.

3. The method of claim 1, wherein the fragment of the 39kDa protein is a 28kDa protein and the amount of 28kDa protein ranges from about 38 to 3,800 mg/kg of body weight/dose.

4. The method of claim 1, wherein the 39kDa protein or fragment thereof is administered 0 to 20 minutes prior to administering the t-PA.

5. The method of claim 1, wherein the hepatic-clearance of t-PA is reduced between 20 and 100%.

6. A 28kDa protein characterized by a molecular weight of 28,000 daltons on SDS-PAGE, stability to acid hydrolysis, solubility in 1% Triton X-100, and possessing biological activity against the t-PA hepatic receptor and reducing hepatic clearance of t-PA between 20 and 100%.

7. A 28kDa protein having an amino acid sequence as set forth in SEQ ID NO: 1:

Tyr	Ser	Arg	Glu	Lys	Asn	Gln	Pro	Lys	Pro	Ser	Pro	Lys	Arg	Glu	Ser
1				5				10					15		
Gly	Glu	Glu	Phe	Arg	Met	Glu	Lys	Leu	Asn	Gln	Leu	Trp	Glu	Lys	Ala
			20					25					30		
Gln	Arg	Leu	His	Leu	Pro	Pro	Val	Arg	Leu	Ala	Glu	Leu	His	Ala	Asp
		35					40					45			
Leu	Lys	Ile	Gln	Glu	Arg	Asp	Glu	Leu	Ala	Trp	Lys	Lys	Leu	Lys	Leu
	50					55					60				

```

Asp Gly Leu Asp Glu Asp Gly Glu Lys Glu Ala Arg Leu Ile Arg Asn
 65          70          75          80
Leu Asn Val Ile Leu Ala Lys Tyr Gly Leu Asp Gly Lys Lys Asp Ala
          85          90          95
Arg Gln Val Thr Ser Asn Ser Leu Ser Gly Thr Gln Glu Asp Gly Leu
          100          105          110
Asp Asp Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly
          115          120          125
Lys Phe Ser Gly Glu Glu Leu Asp Lys Leu Trp Arg Glu Phe Leu His
          130          135          140
His Lys Glu Lys Val His Glu Tyr Asn Val Leu Glu Thr Leu Ser
          145          150          155          160
Arg Thr Glu Glu Ile His Glu Asn Val Ile Ser Pro Ser Asp Leu Ser
          165          170          175
Asp Ile Lys Gly Ser Val Leu His Ser Arg His Thr Glu Leu Lys Glu
          180          185          190
Lys Leu Arg Ser Ile Asn Gln Gly Leu Asp Arg Leu Arg Arg Val Ser
          195          200          205
His Gln Gly Tyr Ser Thr Glu Ala Glu Phe Glu Glu Pro Arg Val Ile
          210          215          220
Asp Leu Trp Asp Leu Ala Gln Ser Ala Asn Leu Thr Asp Lys Glu Leu
          225          230          235          240
Glu Ala Phe Arg Glu Glu Leu Lys His Phe Glu Ala Lys Ile Glu Lys
          245          250          255
His Asn His Tyr Gln Lys Gln Leu Glu Ile Ala His Glu Lys Leu Arg
          260          265          270
His Ala Glu Ser Val Gly Asp Gly Glu Arg Val Ser Arg Ser Arg Glu
          275          280          285
Lys His Ala Leu Leu Glu Gly Arg Thr Lys Glu Leu Gly Tyr Thr Val
          290          295          300
Lys Lys His Leu Gln Asp Leu Ser Gly Arg Ile Ser Arg Ala Arg His
          305          310          315          320
Asn Glu Leu

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8. A chemically synthesized gene encoding a 28kDa protein comprising a chemically synthesized polynucleotide which codes on expression for the amino acid sequence as set forth in SEQ ID NO: 2:

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Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Lys Phe
 1          5          10          15
Ser Gly Glu Glu Leu Asp Lys Leu Trp Arg Glu Phe Leu His His Lys
          20          25          30
Glu Lys Val His Glu Tyr Asn Val Leu Leu Glu Thr Leu Ser Arg Thr
          35          40          45
Glu Glu Ile His Glu Asn Val Ile Ser Pro Ser Asp Leu Ser Asp Ile
          50          55          60
Lys Gly Ser Val Leu His Ser Arg His Thr Glu Leu Lys Glu Lys Leu
          65          70          75          80
Arg Ser Ile Asn Gln Gly Leu Asp Arg Leu Arg Arg Val Ser His Gln
          85          90          95
Gly Tyr Ser Thr Glu Ala Glu Phe Glu Glu Pro Arg Val Ile Asp Leu
          100          105          110

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Trp	Asp	Leu	Ala	Gln	Ser	Ala	Asn	Leu	Thr	Asp	Lys	Glu	Leu	Glu	Ala
		115					120					125			
Phe	Arg	Glu	Glu	Leu	Lys	His	Phe	Glu	Ala	Lys	Ile	Glu	Lys	His	Asn
	130					135					140				
His	Tyr	Gln	Lys	Gln	Leu	Glu	Ile	Ala	His	Glu	Lys	Leu	Arg	His	Ala
145					150					155					160
Glu	Ser	Val	Gly	Asp	Gly	Glu	Arg	Val	Ser	Arg	Ser	Arg	Glu	Lys	His
			165						170					175	
Ala	Leu	Leu	Glu	Gly	Arg	Thr	Lys	Glu	Leu	Gly	Tyr	Thr	Val	Lys	Lys
			180					185					190		
His	Leu	Gln	Asp	Leu	Ser	Gly	Arg	Ile	Ser	Arg	Ala	Arg	His	Asn	Glu
	195					200						205			
Leu															

9. A composition for inhibiting the hepatic clearance of tissue-type plasminogen-activator (t-PA) *in vivo* in humans comprising a t-PA-hepatic clearance-inhibiting amount of 39kDa protein or a t-PA-hepatic clearance inhibiting fragment thereof.

10. The composition of claim 9, wherein the 39kDa protein or fragments thereof is present in a dosage amount of from about 60 to 6,000 mg/kg of body weight/dose.

11. The composition of claim 9, wherein the fragment of the 39kDa protein is a 28kDa protein and is present in a dosage amount of from about 38 to 3,800 mg/kg of body weight/dose.

12. In a pharmaceutical composition for a mammalian patient containing tissue-type plasminogen activator (t-PA), the improvement comprising said composition further including a t-PA hepatic clearance-inhibiting amount of 39kDa protein or a t-PA hepatic clearance-inhibiting fragment thereof.

13. The pharmaceutical composition of claim 12, wherein the t-PA is present in a dosage amount of from about 0.15 to 1.5 mg/kg of body weight/dose.

14. The pharmaceutical composition of claim 12, wherein the 39kDa protein or fragments thereof is present in a dosage amount of from about 60 to 6,000 mg/kg of body weight/dose.

15. The pharmaceutical composition of claim 12, wherein the fragment of the 39kDa protein is a 28kDa protein and is present in a dosage amount of from about 38 to 3,800 mg/kg of body weight/dose.

16. A composition for treating thrombolytic diseases in a mammal comprising an effective amount of tissue-type plasminogen activator (t-PA), and an effective amount of a t-PA hepatic clearance-inhibiting 39kDa protein or a t-PA hepatic clearance-inhibiting fragment thereof.

17. The composition of claim 16, wherein the t-PA is present in a dosage amount of from about 0.15 to 1.5 mg/kg of body weight/dose.

18. The composition of claim 16, wherein the 39kDa protein or fragments thereof is present in a dosage amount of from about 60 to 6,000 mg/kg of body weight/dose.

19. The composition of claim 16, wherein the fragment of the 39kDa protein is a 28kDa protein and is present in a dosage amount of from about 38 to 3,800 mg/kg of body weight/dose.

20. A t-PA-hepatic clearance-inhibiting protein characterized by binding to low density lipoprotein receptor-related protein (LRP) and inhibiting the binding of t-PA to LRP up to about 80%.

21. A t-PA-hepatic clearance-inhibiting protein comprising modified forms of the 39kDa protein and fragments

thereof which bind to LRP and reduce hepatic clearance of t-PA between 20 and 100%.

22. A t-PA-hepatic clearance-inhibiting protein comprising modified forms of the 28kDa protein of claim 7 which bind to LRP and reduce hepatic clearance of t-PA between 20 and 100%.

23. A rat protein which binds to low density lipoprotein receptor-related protein, having an amino acid sequence as set forth in SEQ ID NO: 5:

Pro	Arg	Leu	Glu	Lys	Leu	Trp	His	Lys	Ala	Lys	Thr	Ser	Gly	Ser	Val	1	5	10	15
Arg	Leu	Thr	Ser	Cys	Ala	Arg	Val	Leu	His	Lys	Glu	Lys	Ile	His	Glu	20	25	30	
Tyr	Asn	Val	Leu	Leu	Asp	Thr	Leu	Ser	Arg	Ala	Glu	Glu	Gly	Tyr	Glu	35	40	45	
Asn	Leu	Leu	Ser	Pro	Ser	Asp	Met	Thr	His	Ile	Lys	Ser	Asp	Thr	Leu	50	55	60	
Ala	Ser	Lys	His	Ser	Glu	Leu	Lys	Asp	Arg	Leu	Arg	Ser	Ile	Asn	Gln	65	70	75	80
Gly	Leu	Asp	Arg	Leu	Arg	Lys	Val	Ser	His	Gln	Leu	Arg	Pro	Ala	Thr	85	90	95	
Glu	Phe	Glu	Glu	Pro	Arg	Val	Ile	Asp	Leu	Trp	Asp	Leu	Ala	Gln	Ser	100	105	110	
Ala	Asn	Phe	Thr	Glu	Lys	Glu	Leu	Glu	Ser	Phe	Arg	Glu	Glu	Leu	Lys	115	120	125	
His	Phe	Glu	Ala	Lys	Ile	Glu	Lys	His	Asn	His	Tyr	Gln	Lys	Gln	Leu	130	135	140	
Glu	Ile	Ser	His	Gln	Lys	Leu	Lys	His	Val	Glu	Ser	Ile	Gly	Asp	Pro	145	150	155	160
Glu	His	Ile	Ser	Arg	Asn	Lys	Glu	Lys	Tyr	Val	Leu	Leu	Glu	Glu	Lys	165	170	175	
Thr	Lys	Glu	Leu	Gly	Tyr	Lys	Val	Lys	Lys	His	Leu	Gln	Asp	Leu	Ser	180	185	190	
Ser	Arg	Val	Ser	Arg	Ala	Arg	His	Asn	Glu	Leu						195	200		

24. A chemically synthesized gene encoding a rat protein which binds to low density lipoprotein receptor-related protein comprising a chemically synthesized polynucleotide which codes on expression for the amino acid sequence as set forth in SEQ ID NO: 5:

Pro	Arg	Leu	Glu	Lys	Leu	Trp	His	Lys	Ala	Lys	Thr	Ser	Gly	Ser	Val
1				5					10					15	
Arg	Leu	Thr	Ser	Cys	Ala	Arg	Val	Leu	His	Lys	Glu	Lys	Ile	His	Glu
			20					25					30		
Tyr	Asn	Val	Leu	Leu	Asp	Thr	Leu	Ser	Arg	Ala	Glu	Glu	Gly	Tyr	Glu
		35					40					45			
Asn	Leu	Leu	Ser	Pro	Ser	Asp	Met	Thr	His	Ile	Lys	Ser	Asp	Thr	Leu
	50					55					60				
Ala	Ser	Lys	His	Ser	Glu	Leu	Lys	Asp	Arg	Leu	Arg	Ser	Ile	Asn	Gln
65					70					75					80
Gly	Leu	Asp	Arg	Leu	Arg	Lys	Val	Ser	His	Gln	Leu	Arg	Pro	Ala	Thr
				85					90					95	
Glu	Phe	Glu	Glu	Pro	Arg	Val	Ile	Asp	Leu	Trp	Asp	Leu	Ala	Gln	Ser
			100					105					110		
Ala	Asn	Phe	Thr	Glu	Lys	Glu	Leu	Glu	Ser	Phe	Arg	Glu	Glu	Leu	Lys
		115					120					125			
His	Phe	Glu	Ala	Lys	Ile	Glu	Lys	His	Asn	His	Tyr	Gln	Lys	Gln	Leu
	130					135					140				
Glu	Ile	Ser	His	Gln	Lys	Leu	Lys	His	Val	Glu	Ser	Ile	Gly	Asp	Pro
145					150					155					160
Glu	His	Ile	Ser	Arg	Asn	Lys	Glu	Lys	Tyr	Val	Leu	Leu	Glu	Glu	Lys
				165					170					175	
Thr	Lys	Glu	Leu	Gly	Tyr	Lys	Val	Lys	Lys	His	Leu	Gln	Asp	Leu	Ser
			180					185					190		
Ser	Arg	Val	Ser	Arg	Ala	Arg	His	Asn	Glu	Leu					
		195					200								

1 / 1 8

FIG. 1

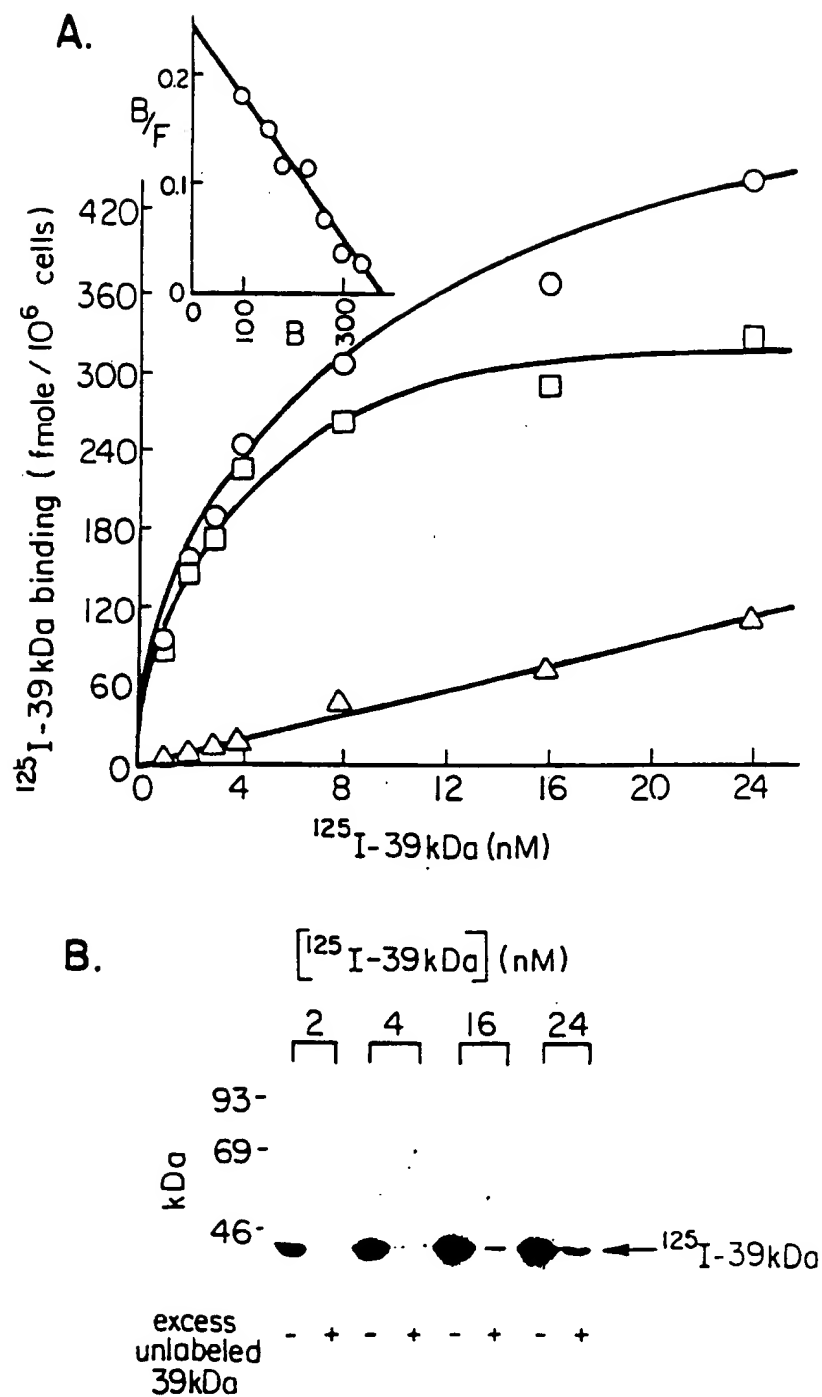


FIG. 2

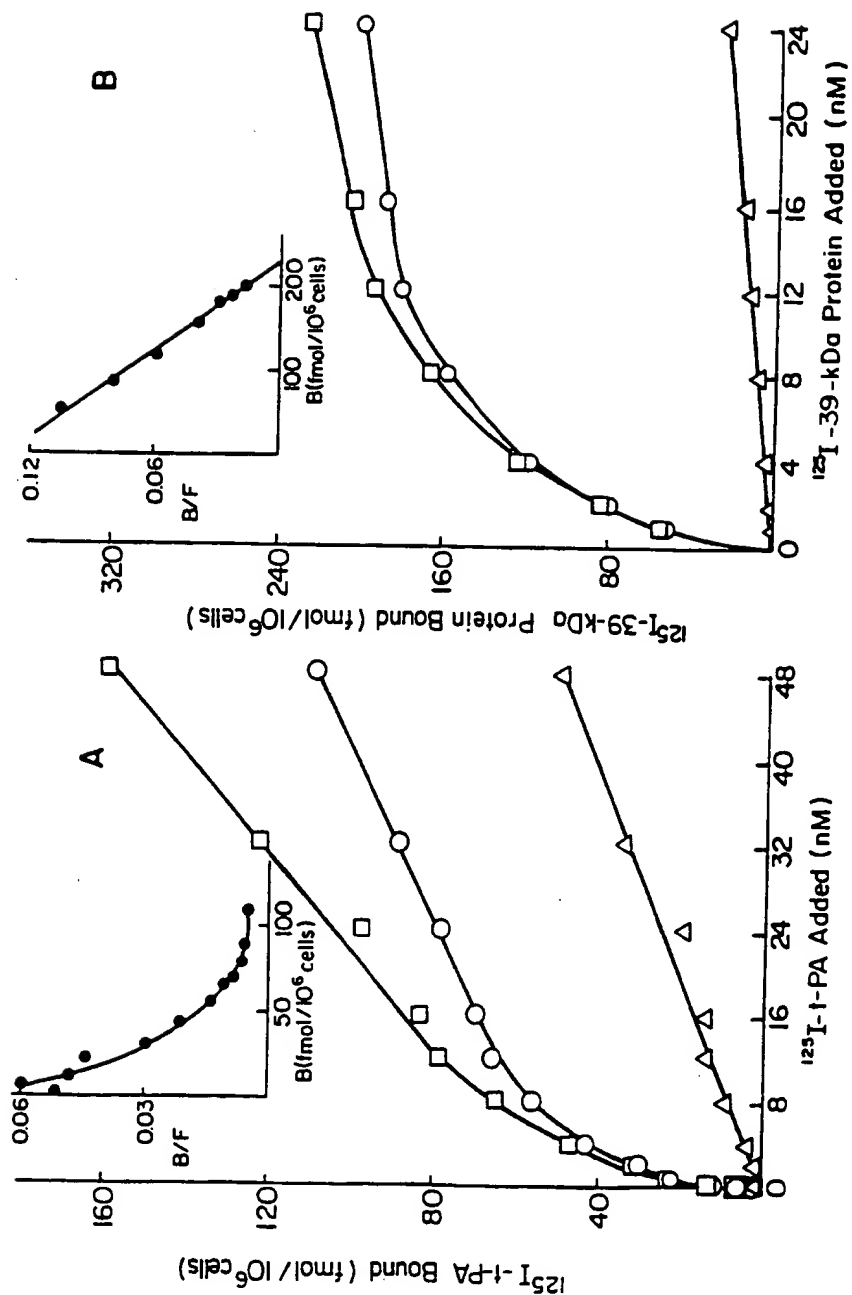
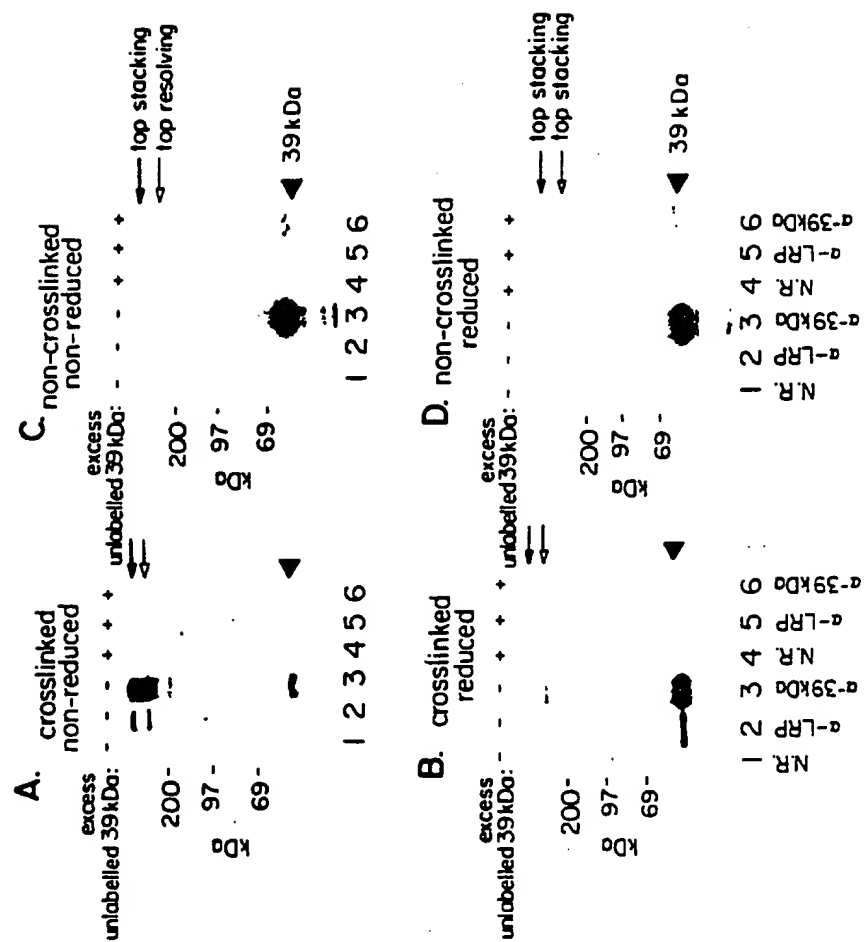
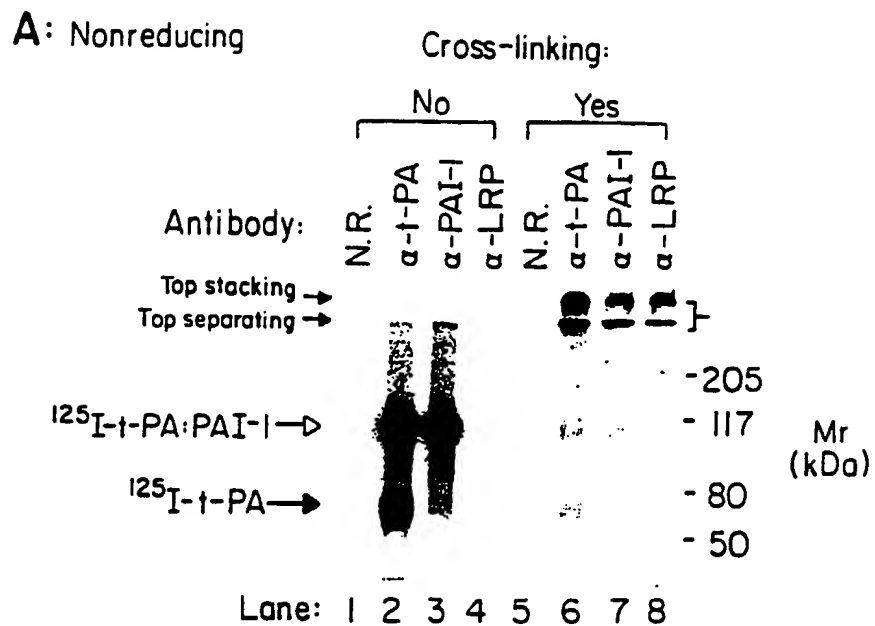


FIG. 3



4 / 1 8

FIG. 4



B: Reducing

Lane: 1 2 3 4 5 6 7 8

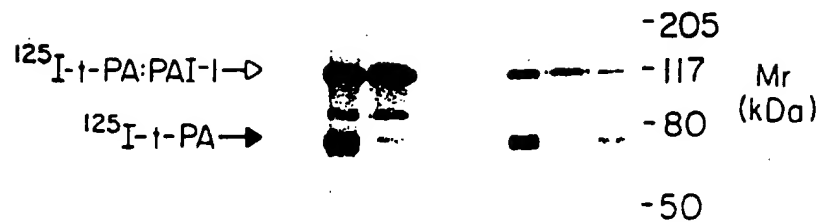
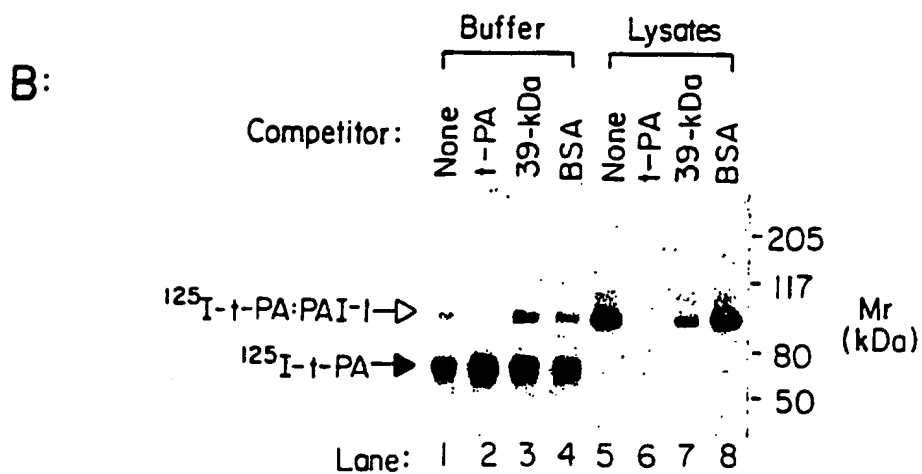
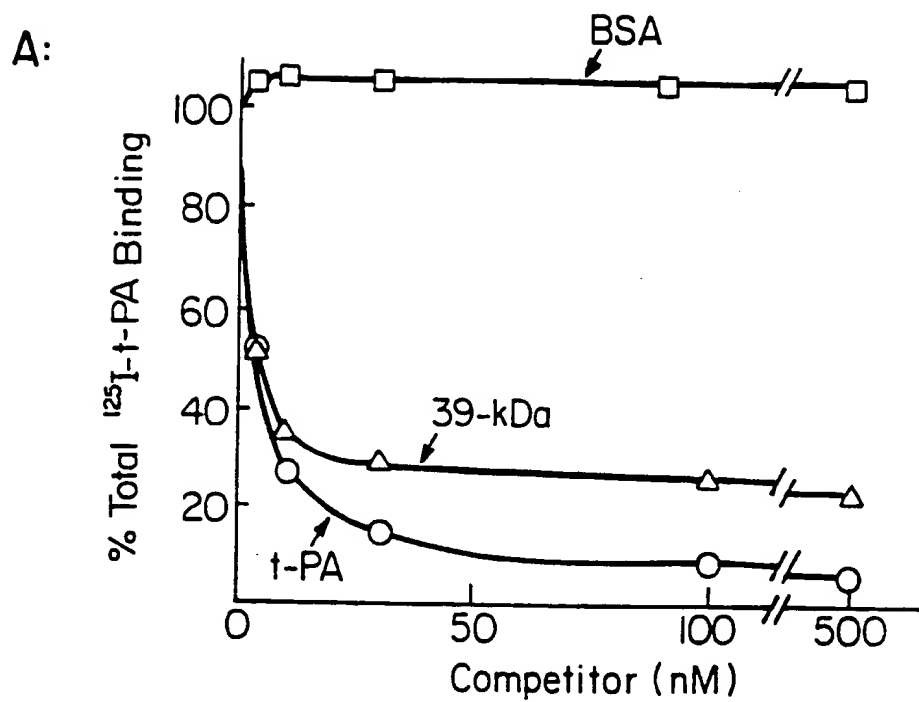


FIG. 5



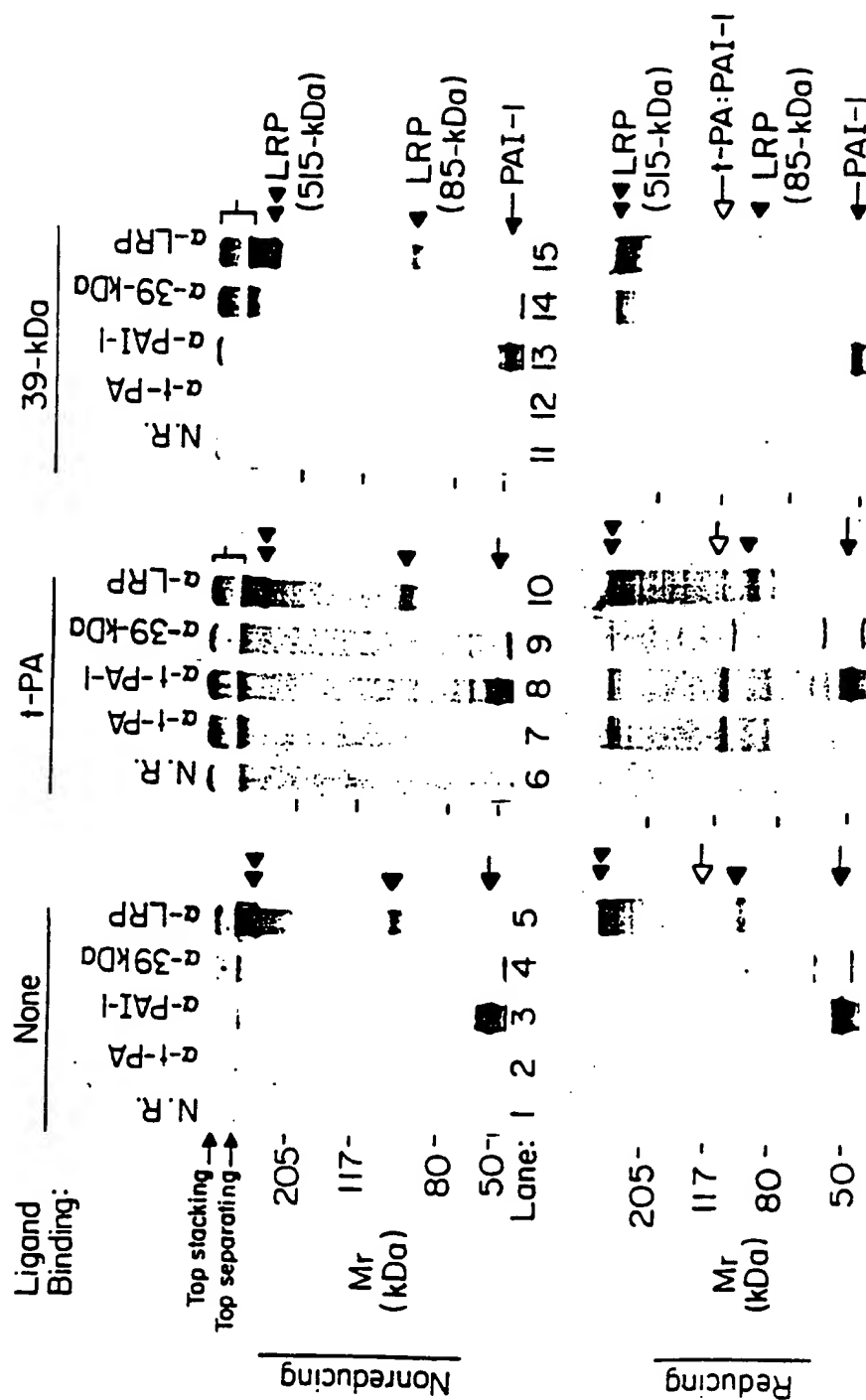
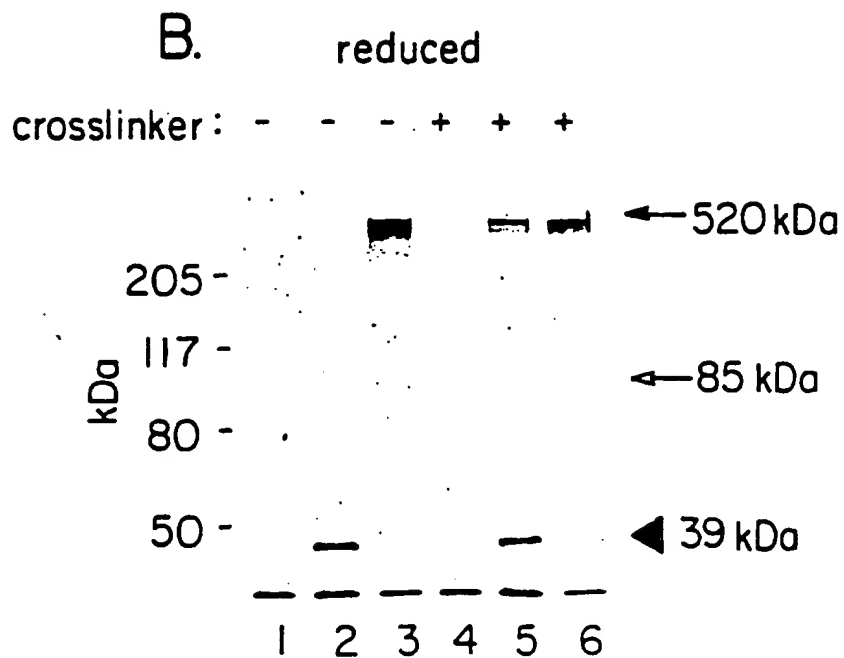
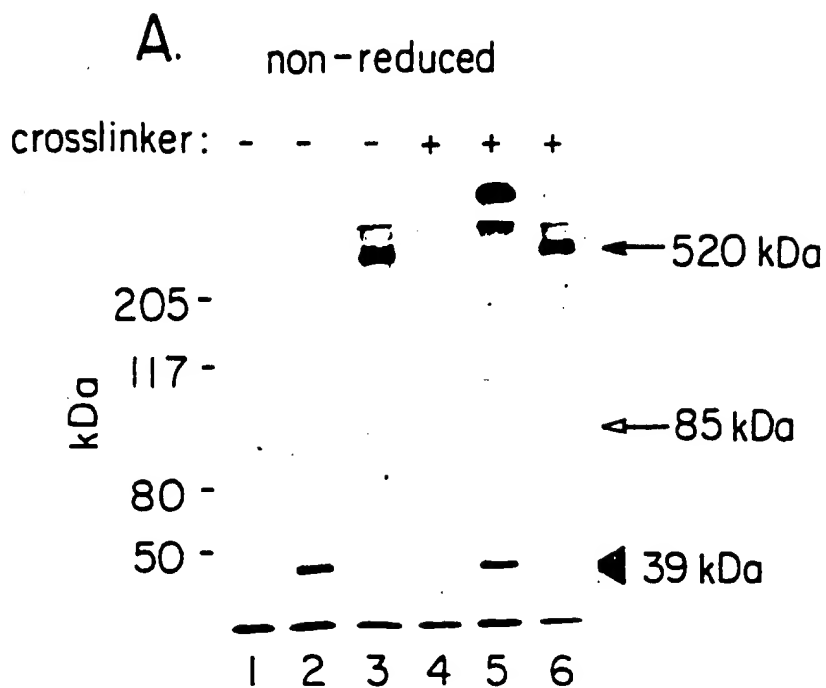


FIG. 6

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FIG. 7



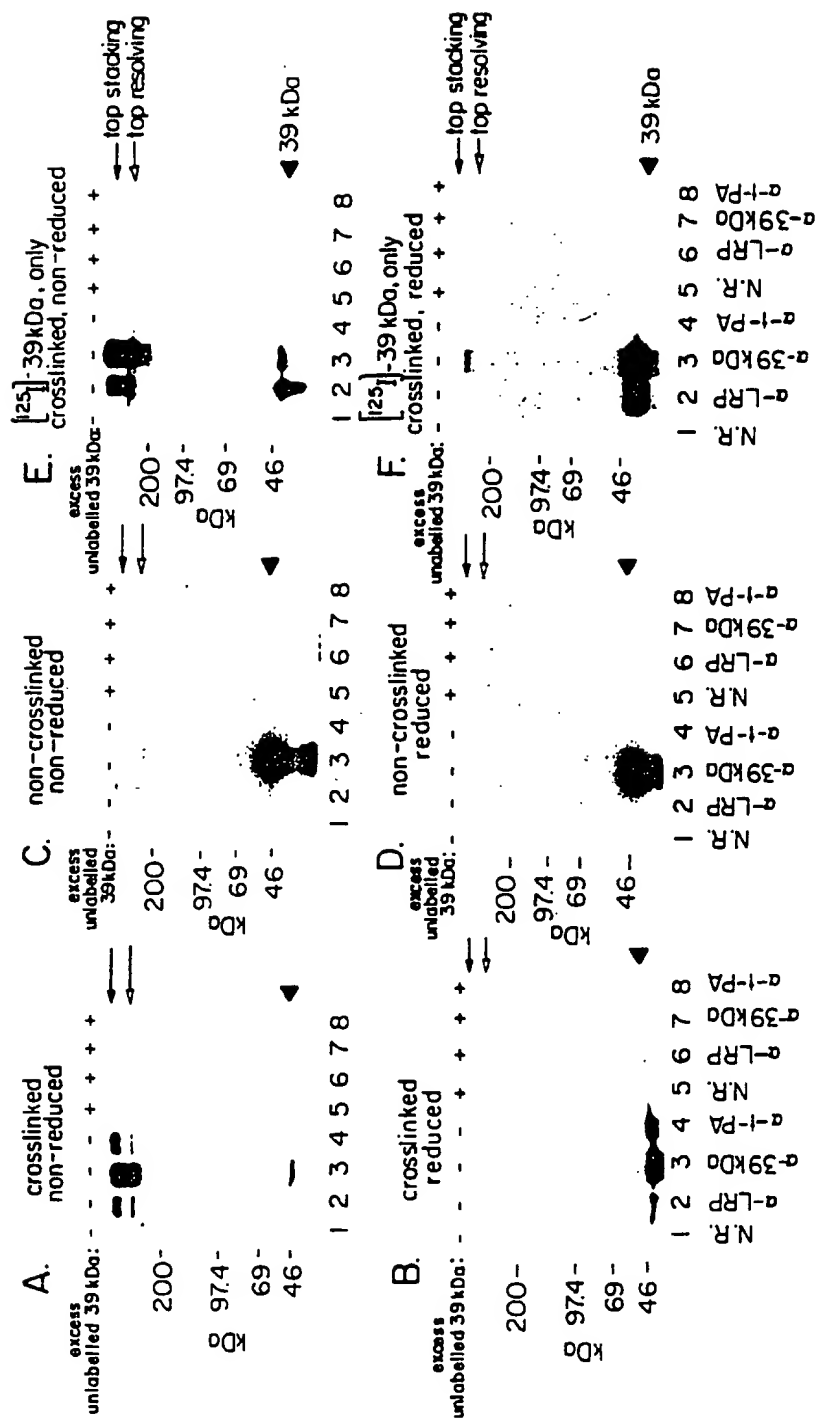
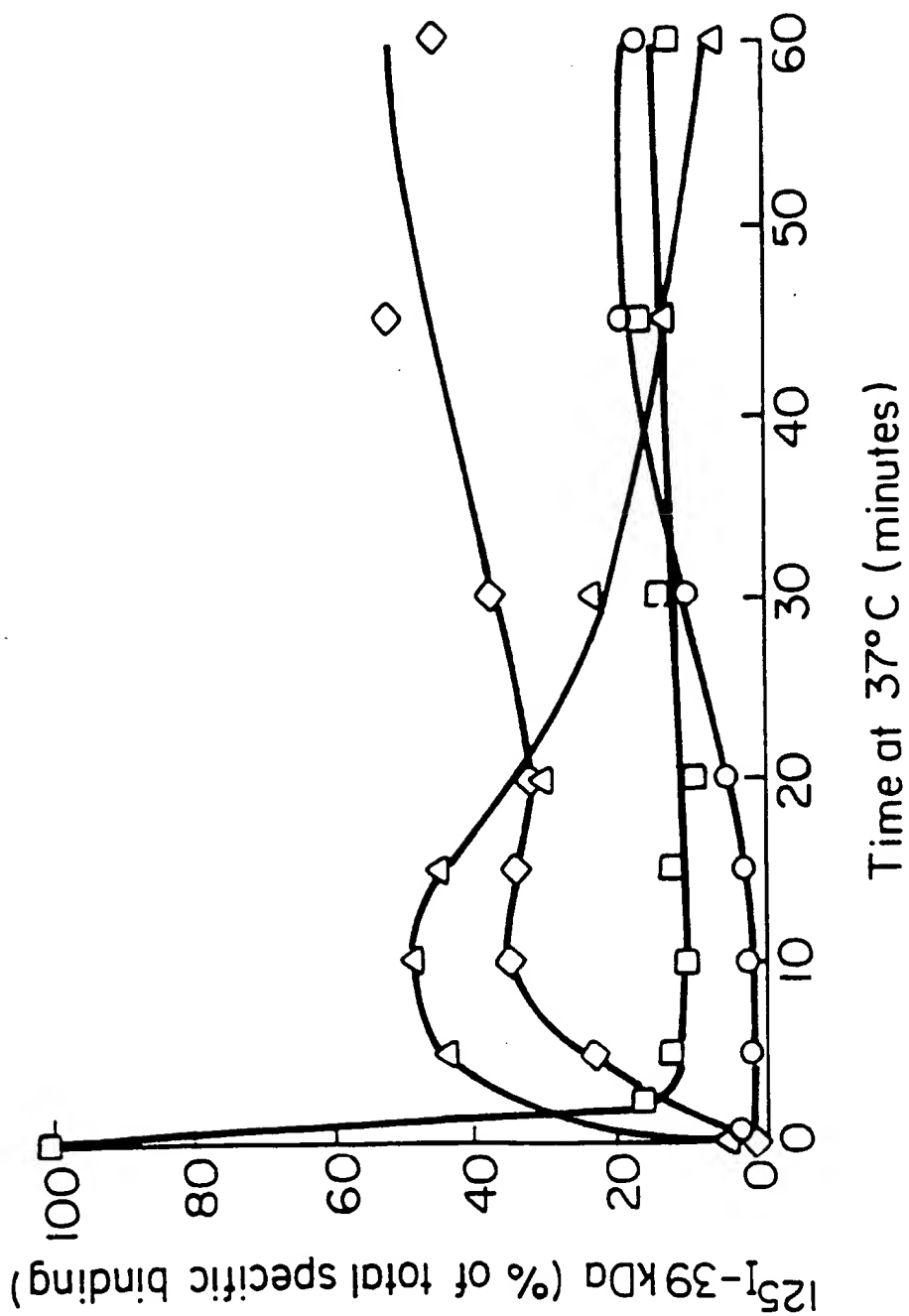


FIG. 8

FIG. 9



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FIG. 10

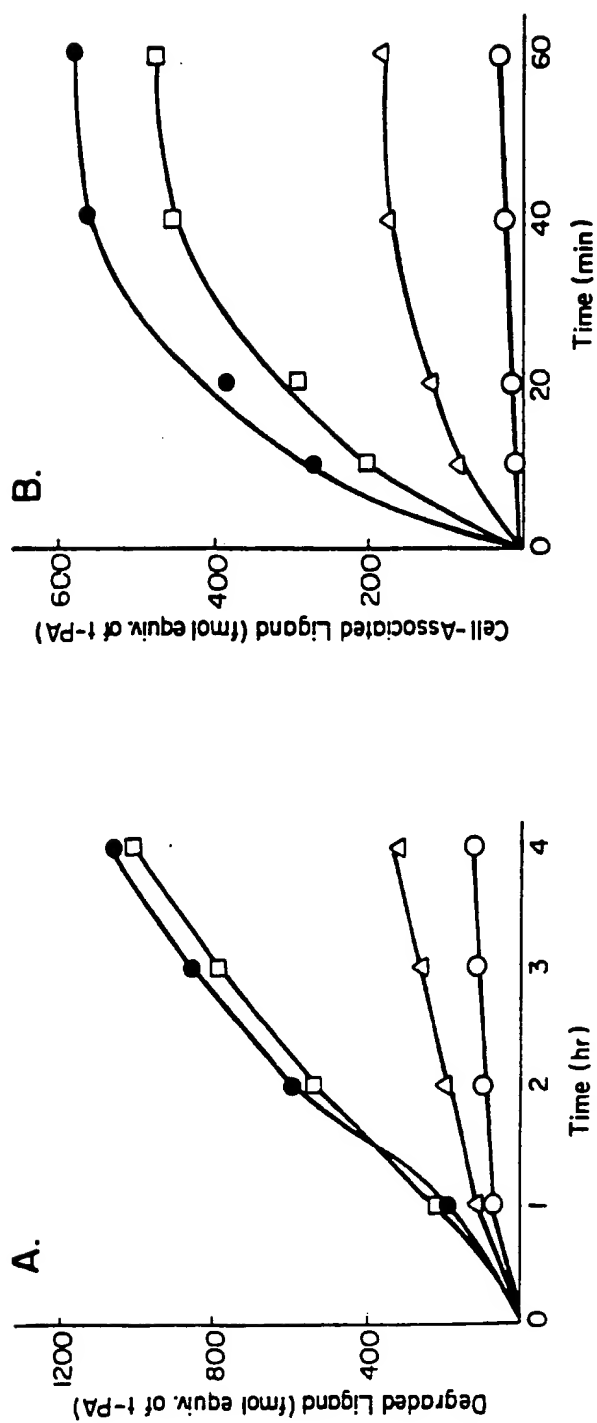


FIG. 11

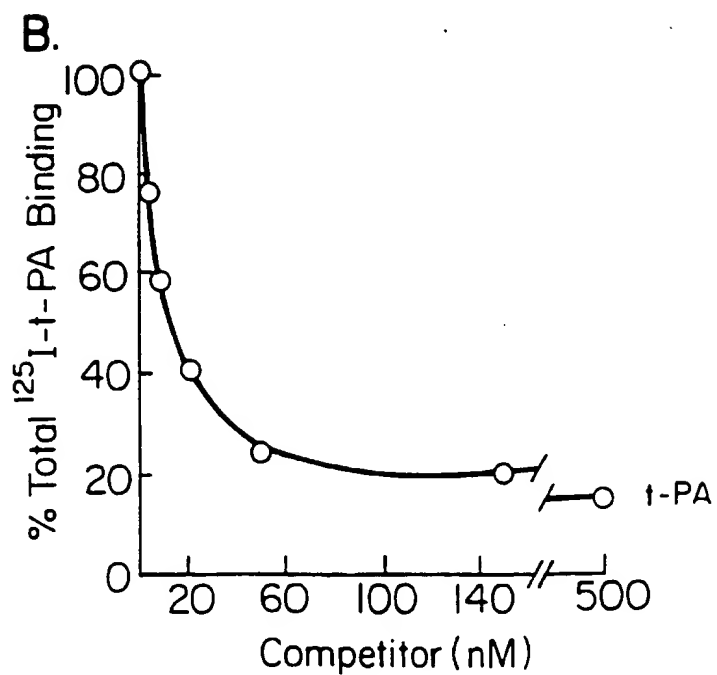
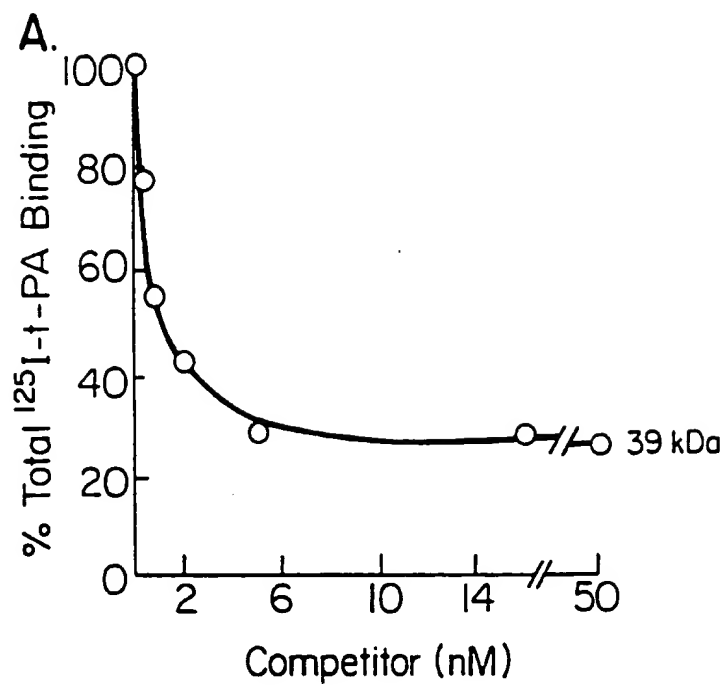


FIG. 12

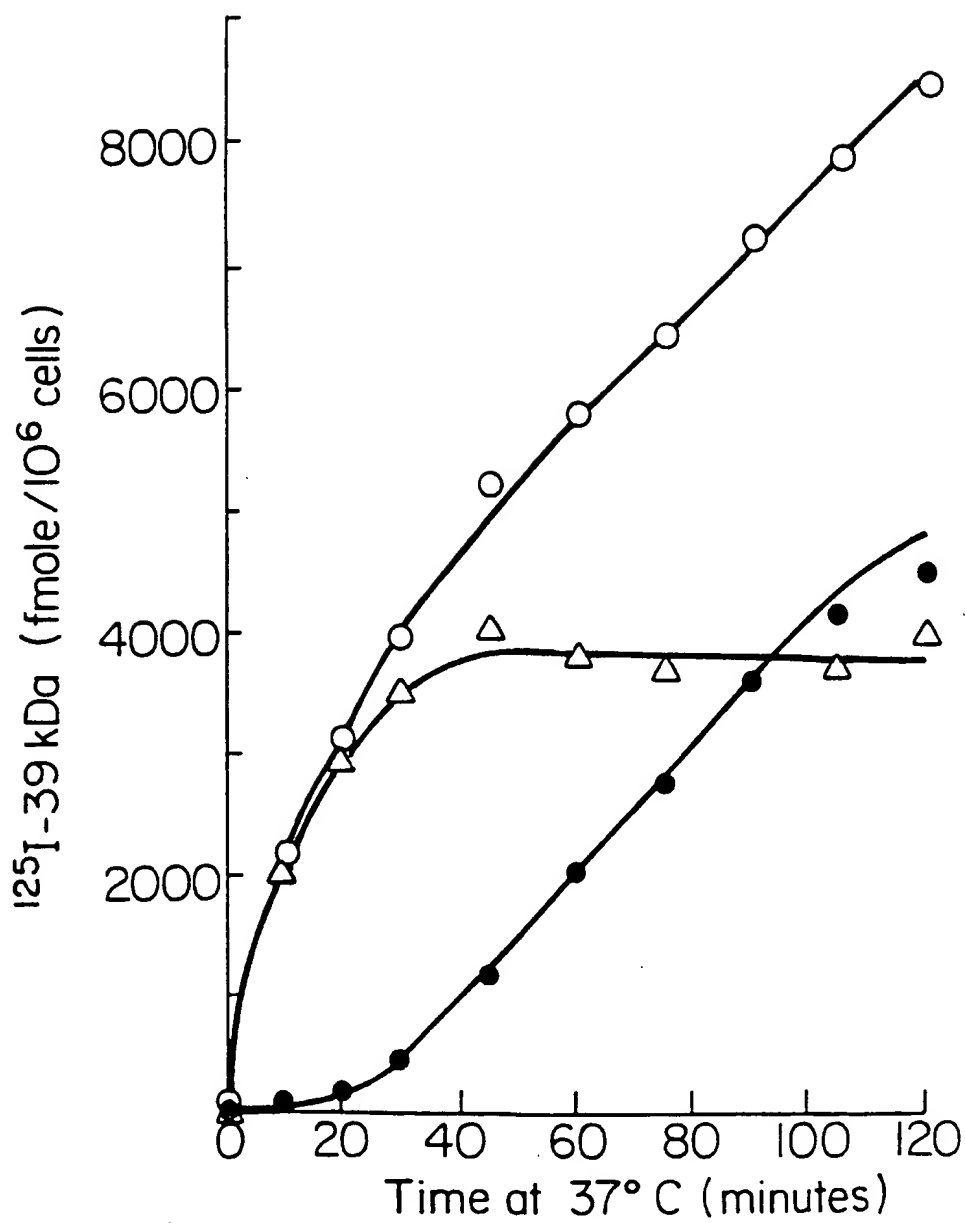
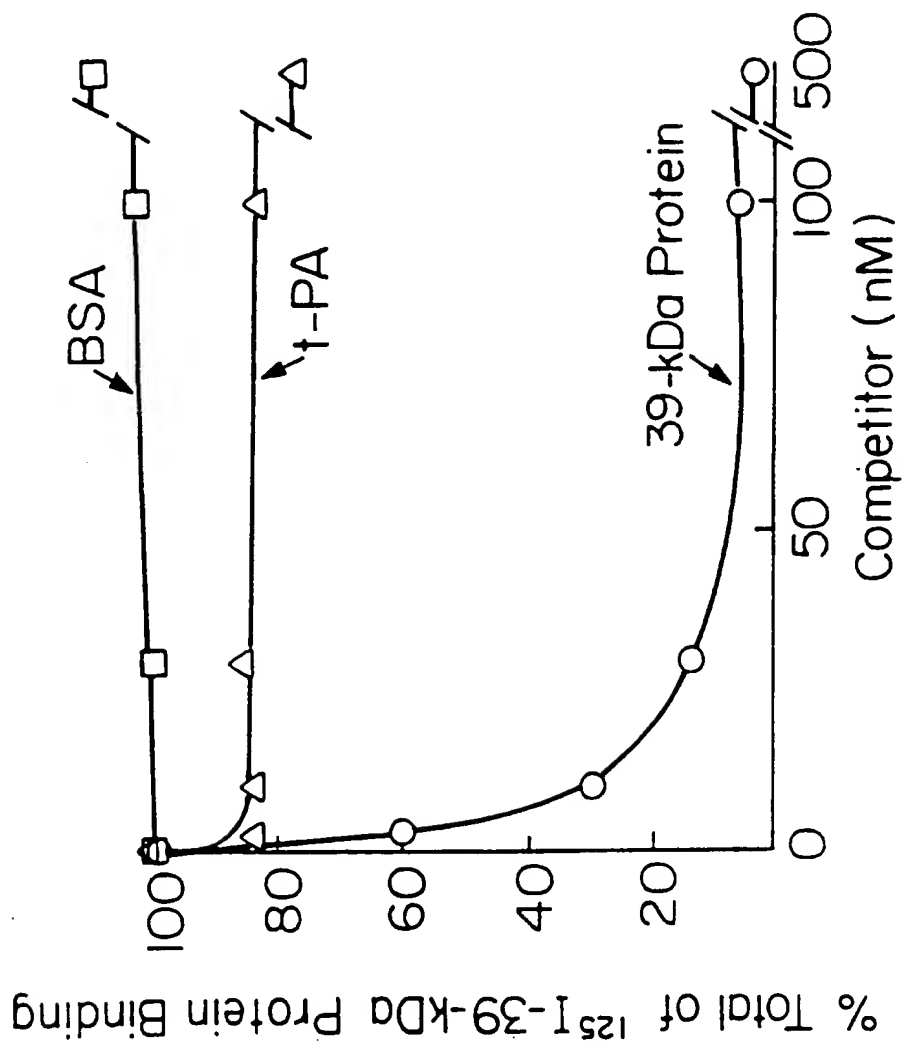


FIG. 13



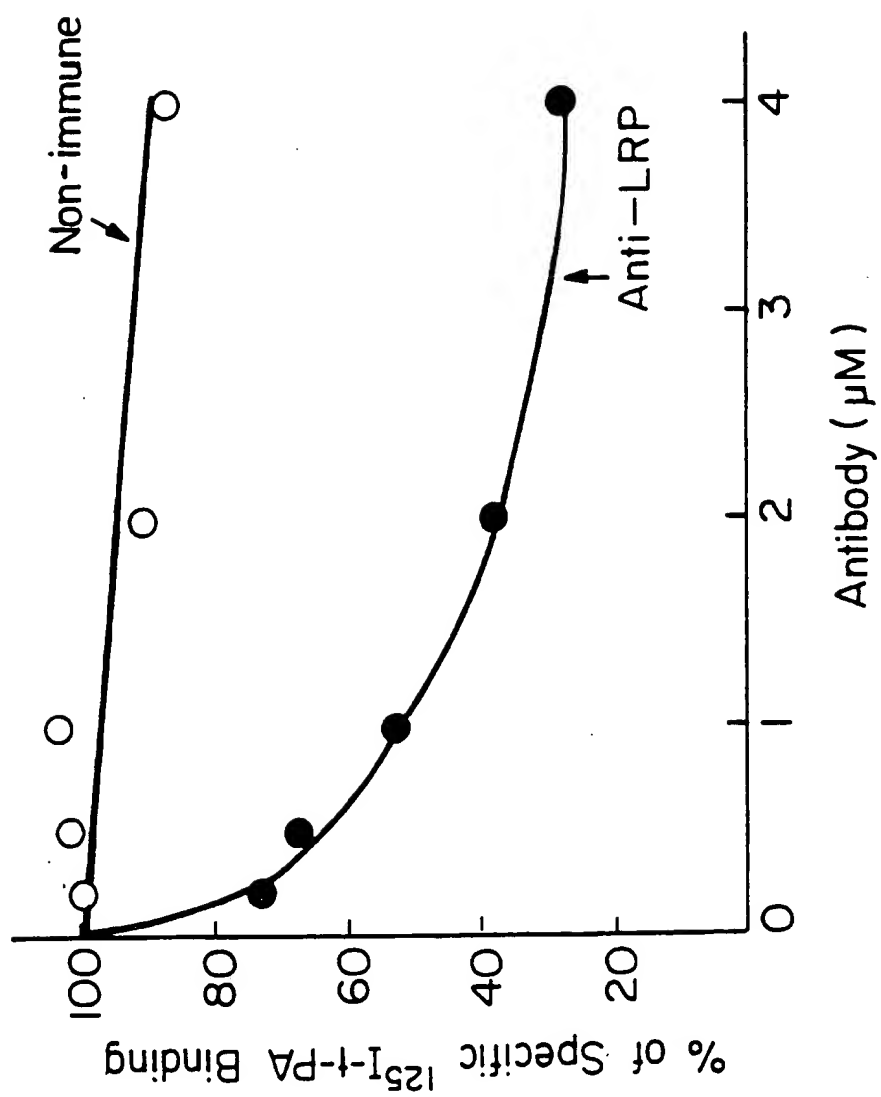
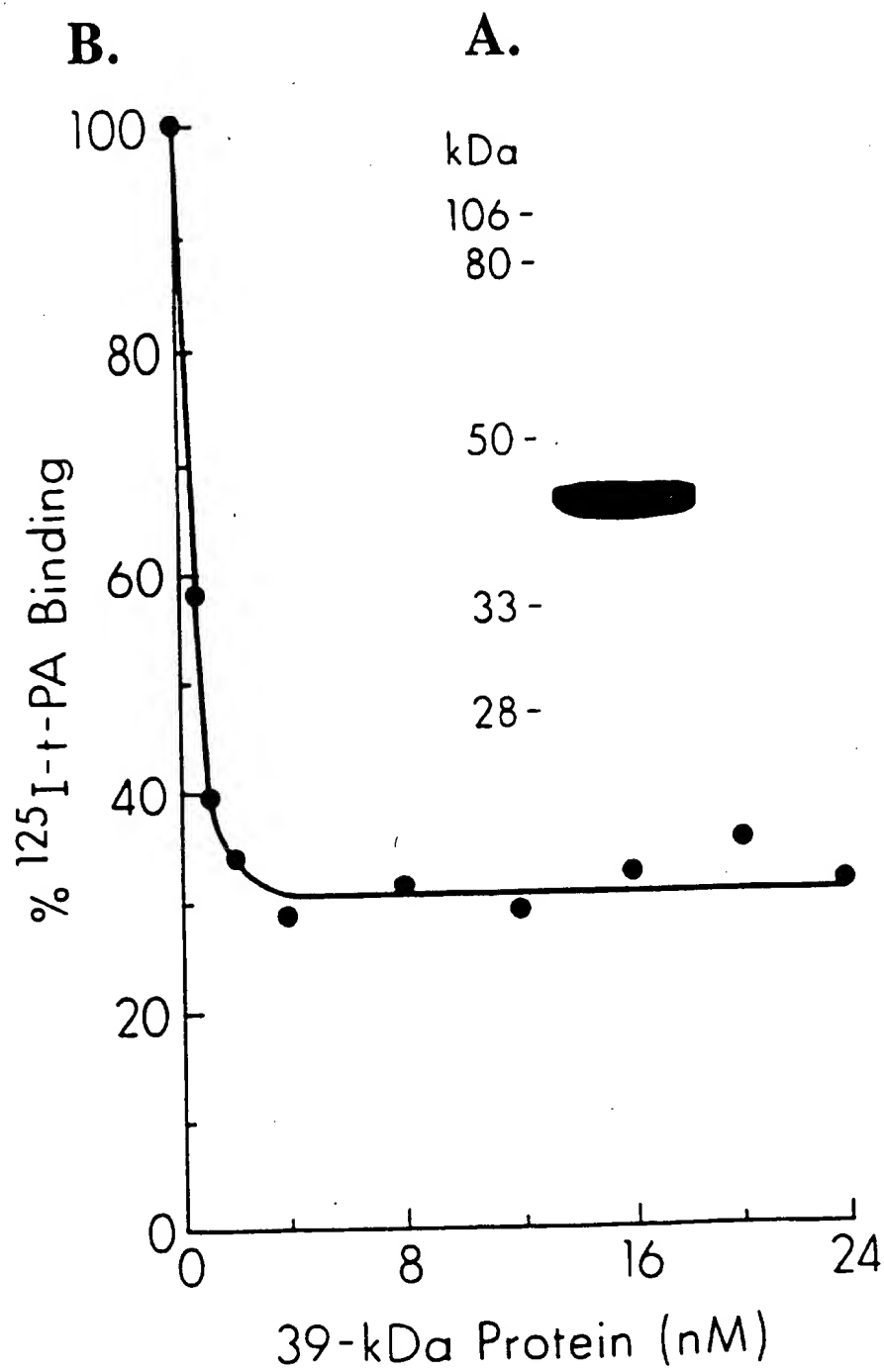
**FIG. 14**

FIG. 15



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FIG. 16

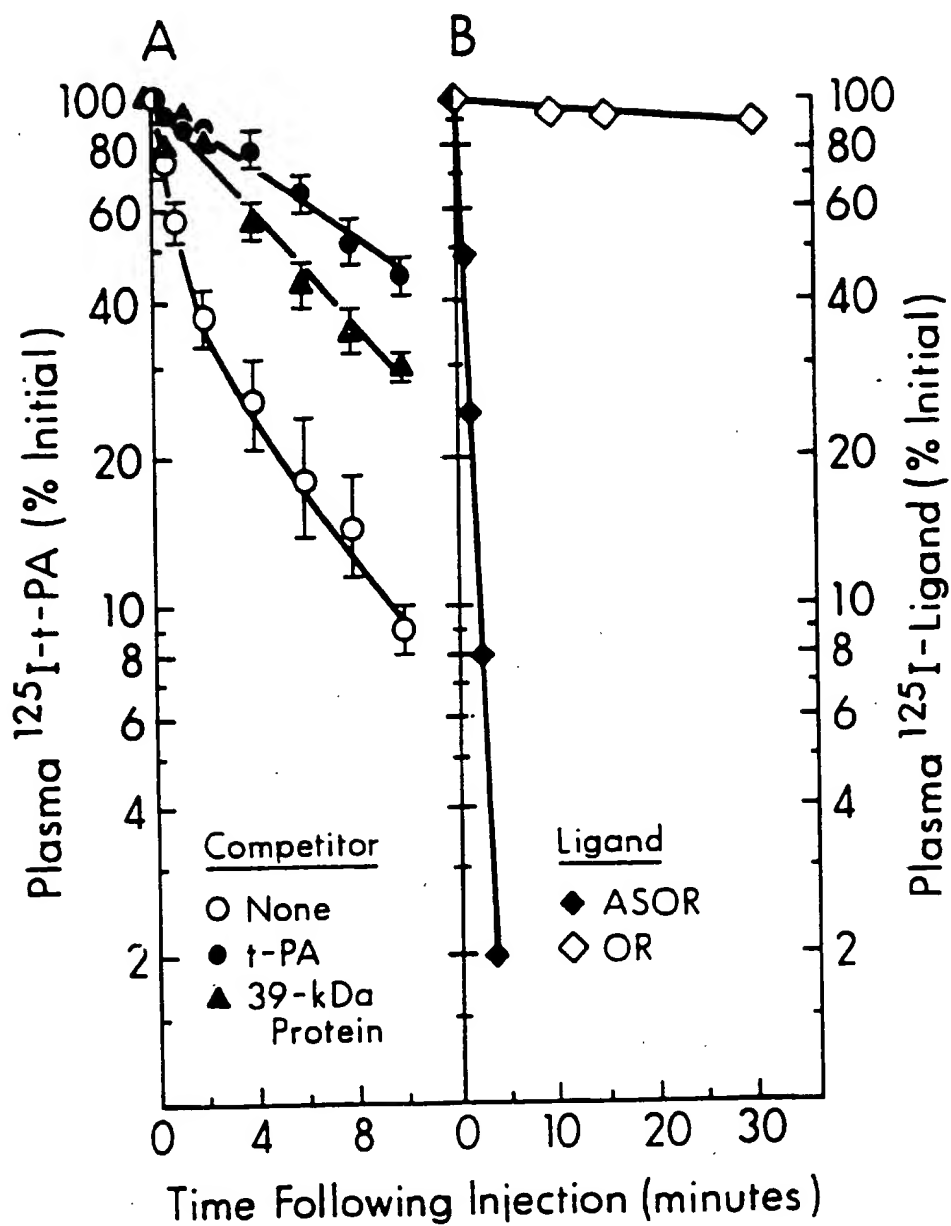


FIG. 17

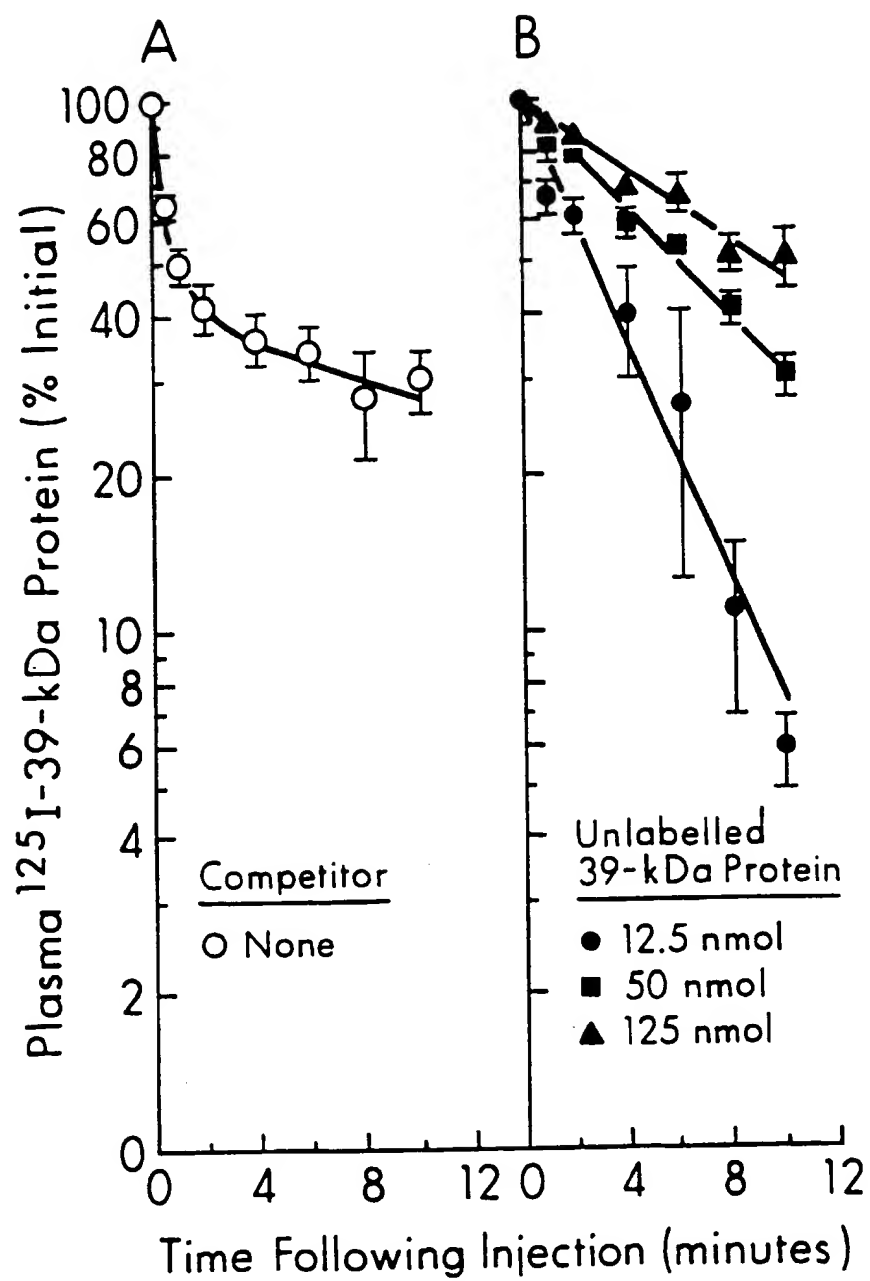
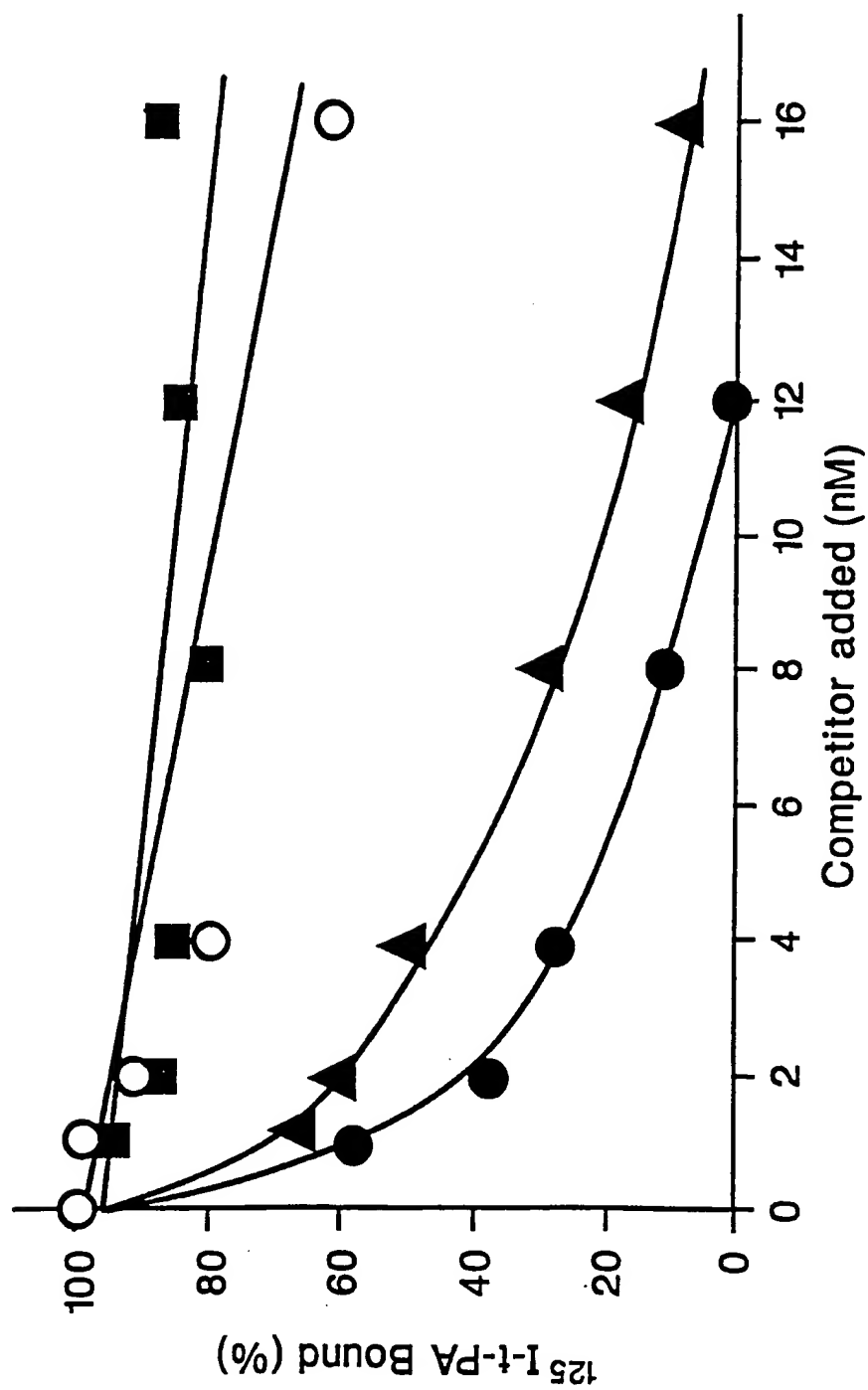


FIG. 18
Inhibition of ^{125}I -t-PA Binding to Rat Liver MH_1C_1 Cells by
39 kDa Protein, 20 kDa N-, and 28 kDa C-terminal Fragment



INTERNATIONAL SEARCH REPORT

National Application No
PCT/US 93/12380

A. CLASSIFICATION OF SUBJECT MATTER

A 61 K 37/47, A 61 K 37/43, C 07 H 21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A 61 K, C 07 H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 267, no. 22, issued 1992, August 05 The American Society of Biochemistry and Molecular Biology, Baltimore G. BU et al.: "Identification and Partial Characterization by Chemical Cross-linking of a Binding Protein for the Tissue-type Plasminogen Activator (t-PA) on Rat Hepatoma Cells", pages 15595-15602, abstract.	1-8, 20-23
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 266, no. 31, issued 1991, November 05	1-8, 20-23

☐ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

04 May 1994

Date of mailing of the international search report

25 -05- 1994

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Fax (+ 31-70) 340-3016

Authorized officer

SCHNASS e.h.

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	<p>The American Society of Biochemistry and Molecular Biology, Baltimore</p> <p>J. HERZ et al. "39-kDa Protein Modulates Binding of Ligands to Low Density Lipoprotein Receptor-related Protein/alpha 2 - Macroglobulin Receptor", pages 21232-21238, abstract.</p> <p>--</p>	
A	<p>THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, no. 22, issued 1988, August 05</p> <p>The American Society of Biochemistry and Molecular Biology, Baltimore</p> <p>D.A. OWENSBY et al. "Receptor-mediated Endocytosis of Tissue-type Plasminogen Activator by the Human Hepatoma Cell Line Hep G2", pages 10587-10594, abstract.</p> <p>--</p>	1
A	<p>THROMBOSIS AND HAEMOSTASIS, vol. 54, no. 01, issued 1985, July 14</p> <p>M. EINARSSON et al. "Endocytosis of Tissue Plasminogen Activator (t-PA) by Rat Liver Cells", page 270, abstract no. P1601.</p> <p>--</p>	1
A	<p>WO, A1, 84/01 960 (BEECHAM GROUP P.L.C.) 24 May 1984 (24.05.84), abstract; page 14, example 5.</p> <p>----</p>	1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 93/12380

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-5
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-5 are directed to a therapeutical method of treatment for human body (Rule 39.1(iv)PCT) the search has been carried out.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANHANG

zum internationalen Recherchen-
bericht über die internationale
Patentanmeldung Nr.

ANNEX

to the International Search
Report to the International Patent
Application No.

ANNEXE

au rapport de recherche inter-
national relatif à la demande de brevet
international n°

PCT/US 93/12380 SAE B4484

In diesem Anhang sind die Mitglieder
der Patentfamilien der im obenge-
nannten internationalen Recherchenbericht
angeführten Patentdokumente angegeben.
Diese Angaben dienen nur zur Unter-
richtung und erfolgen ohne Gewähr.

This Annex lists the patent family
members relating to the patent documents
cited in the above-mentioned inter-
national search report. The Office is
in no way liable for these particulars
which are given merely for the purpose
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La présente annexe indique les
membres de la famille de brevets
relatifs aux documents de brevets cités
dans le rapport de recherche inter-
national visée ci-dessus. Les renseigne-
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In Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WO A1 8401960	24-05-84	EP A1 125269	21-11-84